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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Nicholas C. Nicolaides, Luigi Grasso, and
Philip M. Sass

Serial No.: Not assigned **Group Art Unit:** Not assigned

Filing Date: November 7, 2000 **Examiner:** Not assigned

For: METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODY-PRODUCING CELL LINES WITH IMPROVED ANTIBODY CHARACTERISTICS

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☐ Provisional ☐ Design

Assistant Commissioner for Patents
Washington DC 20231

Sir:

PATENT APPLICATION TRANSMITTAL LETTER

Transmitted herewith for filing, please find

☒ A Utility Patent Application under 37 C.F.R. 1.53(b).

It is a continuing application, as follows:

☐ continuation ☐ divisional ☐ continuation-in-part of prior application number ___/_____.

☐ A Provisional Patent Application under 37 C.F.R. 1.53(c).

☐ A Design Patent Application (submitted in duplicate).

Including the following:

☐ Provisional Application Cover Sheet.

☒ New or Revised Specification, including pages 1 to 41 containing:

- ☒ Specification
- ☒ Claims
- ☒ Abstract
- ☐ Substitute Specification, including Claims and Abstract.

  - ☐ The present application is a continuation application of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Since none of those amendments incorporate new matter into the parent application, the present revised Specification also does not include new matter.

  - ☐ The present application is a continuation application of Application No. _____ filed _____, which in turn is a continuation-in-part of Application No. _____ filed _____. The present application includes the Specification of the parent application which has been revised in accordance with the amendments filed in the parent application. Although the amendments in the parent C-I-P application may have incorporated new matter, since those are the only revisions included in the present application, the present application includes no new matter in relation to the parent application.

☐ A copy of earlier application Serial No. _____ Filed _____, including Specification, Claims and Abstract (pages 1 - @@), to which no new matter has been added TOGETHER WITH a copy of the executed oath or declaration for such earlier application and all drawings and appendices. Such earlier application is hereby incorporated into the present application by reference.

☐ Please enter the following amendment to the Specification under the Cross-Reference to Related Applications section (or create such a section) : "This Application:
☐ is a continuation of ☐ is a divisional of ☐ claims benefit of U.S. provisional Application Serial No. _____filed _____

_____

_____

_____.

☐ Signed Statement attached deleting inventor(s) named in the prior application.

☐ A Preliminary Amendment.

☒ 7 Sheets of ☒ Formal ☐ Informal Drawings.

☐ Petition to Accept Photographic Drawings.

☐ Petition Fee

☒ An ☐ Executed ☒ Unexecuted Declaration or Oath and Power of Attorney.

☐ An Associate Power of Attorney.

☐ An ☐ Executed ☐ Copy of Executed Assignment of the Invention to _____

_____.

☐ A Recordation Form Cover Sheet.

☐ Recordation Fee - $40.00.

☐ The prior application is assigned of record to _____

☐ Priority is claimed under 35 U.S.C. § 119 of Patent Application No. _____ filed _____ in _____ (country).

☐ A Certified Copy of each of the above applications for which priority is claimed:

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____.

☐ An ☐ Executed or ☐ Copy of Executed Earlier Statement Claiming Small Entity Status under 37 C.F.R. 1.9 and 1.27

☐ is enclosed.

☐ has been filed in prior application Serial No. _____ filed _____, said status is still proper and desired in present case.

☒ Diskette Containing DNA/Amino Acid Sequence Information.

☒ Statement to Support Submission of DNA/Amino Acid Sequence Information.

☐ The computer readable form in this application _____, is identical with that filed in Application Serial Number _____, filed _____. In accordance with 37 CFR 1.821(e), please use the ☐ first-filed, ☐ last-filed or ☐ only computer readable form filed in that application as the computer readable form for the instant application. It is understood that the Patent and Trademark Office will make the necessary change in application number and filing date for the computer readable form that will be used for the instant application. A paper copy of the Sequence Listing is ☐ included in the originally-filed specification of the instant application, ☐ included in a separately filed preliminary amendment for incorporation into the specification.

☐ Information Disclosure Statement.

- ☐ Attached Form 1449.
- ☐ Copies of each of the references listed on the attached Form PTO-1449 are enclosed herewith.

☐ A copy of Petition for Extension of Time as filed in the prior case.

☐ Appended Material as follows: _____.

☒ Return Receipt Postcard (should be specifically itemized).

☐ Other as follows: _____

_____

_____.

FEE CALCULATION:

☐ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

			SMALL ENTITY		NOT SMALL ENTITY	
			RATE	FEE	RATE	FEE
PROVISIONAL APPLICATION			$75.00	$	$150.00	$
DESIGN APPLICATION			$160.00	$	$320.00	$
UTILITY APPLICATIONS BASE FEE			$355.00	$	$710.00	$710.00
UTILITY APPLICATION; ALL CLAIMS CALCULATED AFTER ENTRY OF ALL AMENDMENTS						
	No. Filed	No. Extra				
TOTAL CLAIMS	72 - 20 =	52	$9 each	$	$18 each	$936.00
INDEP. CLAIMS	7 - 3 =	4	$40 each	$	$80 each	$320.00
FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM			$135	$	$270	$
ADDITIONAL FILING FEE				$		$
TOTAL FILING FEE DUE				$		$1,966.00

☒ A Check is enclosed in the amount of $ 1,966.00 .

☒ The Commissioner is authorized to charge payment of the following fees and to refund any overpayment associated with this communication or during the pendency of this application to deposit account 23-3050. This sheet is provided in duplicate.

☐ The foregoing amount due.

☒ Any additional filing fees required, including fees for the presentation of extra claims under 37 C.F.R. 1.16.

☒ Any additional patent application processing fees under 37 C.F.R. 1.17 or 1.20(d).

☐ The issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance.

☒ The Commissioner is hereby requested to grant an extension of time for the appropriate length of time, should one be necessary, in connection with this filing or any future filing submitted to the U.S. Patent and Trademark Office in the above-

identified application during the pendency of this application. The Commissioner is further authorized to charge any fees related to any such extension of time to deposit account 23-3050. This sheet is provided in duplicate.

SHOULD ANY DEFICIENCIES APPEAR with respect to this application, including deficiencies in payment of fees, missing parts of the application or otherwise, the United States Patent and Trademark Office is respectfully requested to promptly notify the undersigned.

Date: 11/7/00

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METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODY-PRODUCING CELL LINES WITH IMPROVED ANTIBODY CHARACTERISTICS

TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of antibody maturation and cellular production. In particular, it is related to the field of mutagenesis.

BACKGROUND OF THE INVENTION

The use of antibodies to block the activity of foreign and/or endogenous polypeptides provides an effective and selective strategy for treating the underlying cause of disease. In particular is the use of monoclonal antibodies (MAb) as effective therapeutics such as the FDA approved ReoPro (Glaser, V. (1996) Can ReoPro repolish tarnished monoclonal therapeutics? *Nat. Biotechnol.* 14:1216-1217), an anti-platelet MAb from Centocor; Herceptin (Weiner, L.M. (1999) Monoclonal antibody therapy of cancer. *Semin. Oncol.* 26:43-51), an anti-Her2/neu MAb from Genentech; and Synagis (Saez-Llorens, X.E., *et al.* (1998) Safety and pharmacokinetics of an intramuscular humanized monoclonal antibody to respiratory syncytial virus in premature infants and infants with bronchopulmonary dysplasia. *Pediat. Infect. Dis. J.* 17:787-791), an anti-respiratory syncytial virus MAb produced by Medimmune.

Standard methods for generating MAbs against candidate protein targets are known by those skilled in the art. Briefly, rodents such as mice or rats are injected with a purified antigen in the presence of adjuvant to generate an immune response (Shield, C.F., *et al.* (1996) A cost-effective analysis of OKT3 induction therapy in cadaveric kidney

transplantation. *Am. J. Kidney Dis.* 27:855-864). Rodents with positive immune sera are sacrificed and splenocytes are isolated. Isolated splenocytes are fused to melanomas to produce immortalized cell lines that are then screened for antibody production. Positive lines are isolated and characterized for antibody production. The direct use of rodent MAbs as human therapeutic agents were confounded by the fact that human anti-rodent antibody (HARA) responses occurred in a significant number of patients treated with the rodent-derived antibody (Khazaeli, M.B., *et al.*, (1994) Human immune response to monoclonal antibodies. *J. Immunother.* 15:42-52). In order to circumvent the problem of HARA, the grafting of the complementarity determining regions (CDRs), which are the critical motifs found within the heavy and light chain variable regions of the immunoglobulin (Ig) subunits making up the antigen binding domain, onto a human antibody backbone found these chimeric molecules are able to retain their binding activity to antigen while lacking the HARA response (Emery, S.C., and Harris, W.J. "Strategies for humanizing antibodies" In: ANTIBODY ENGINEERING C.A.K. Borrebaeck (Ed.) Oxford University Press, N.Y. 1995. pp. 159-183. A common problem that exists during the "humanization" of rodent-derived MAbs (referred to hereon as HAb) is the loss of binding affinity due to conformational changes in the 3 dimensional structure of the CDR domain upon grafting onto the human Ig backbone (U.S. Patent No. 5,530,101 to Queen *et al.*). To overcome this problem, additional HAb vectors are usually needed to be engineeredby inserting or deleting additional amino acid residues within the framework region and/or within the CDR coding region itself in order to recreate high affinity HAbs (U.S. Patent No. 5,530,101 to Queen *et al.*). This process is a very time consuming procedure that involves the use of expensive computer modeling programs to predict changes that may lead to a high affinity HAb. In some instances the affinity of the HAb is never restored to that of the MAb, rendering them of little therapeutic use.

Another problem that exists in antibody engineering is the generation of stable, high yielding producer cell lines that is required for manufacturing of the molecule for clinical materials. Several strategies have been adopted in standard practice by those skilled in the art to circumvent this problem. One method is the use of Chinese Hamster Ovary (CHO) cells transfected with exogenous Ig fusion genes containing the grafted human light and heavy

chains to produce whole antibodies or single chain antibodies, which are a chimeric molecule containing both light and heavy chains that form an antigen-binding polypeptide (Reff, M.E. (1993) High-level production of recombinant immunoglobulins in mammalian cells. *Curr. Opin. Biotechnol.* 4:573-576). Another method employs the use of human lymphocytes derived from transgenic mice containing a human grafted immune system or transgenic mice containing a human Ig gene repertoire. Yet another method employs the use of monkeys to produce primate MAbs, which have been reported to lack a human anti-monkey response (Neuberger, M., and Gruggermann, M. (1997) Monoclonal antibodies. Mice perform a human repertoire. *Nature* 386:25-26). In all cases, the generation of a cell line that is capable of generating sufficient amounts of high affinity antibody poses a major limitation for producing sufficient materials for clinical studies. Because of these limitations, the utility of other recombinant systems such as plants are currently being explored as systems that will lead to the stable, high-level production of humanized antibodies (Fiedler, U., and Conrad, U. (1995) High-level production and long-term storage of engineered antibodies in transgenic tobacco seeds. *Bio/Technology* 13:1090-1093).

A method for generating diverse antibody sequences within the variable domain that results in HAbs and MAbs with high binding affinities to antigens would be useful for the creation of more potent therapeutic and diagnostic reagents respectively. Moreover, the generation of randomly altered nucleotide and polypeptide residues throughout an entire antibody molecule will result in new reagents that are less antigenic and/or have beneficial pharmacokinetic properties. The invention described herein is directed to the use of random genetic mutation throughout an antibody structure *in vivo* by blocking the endogenous mismatch repair (MMR) activity of a host cell producing immunoglobulins that encode biochemically active antibodies. The invention also relates to methods for repeated *in vivo* genetic alterations and selection for antibodies with enhanced binding and pharmacokinetic profiles.

In addition, the ability to develop genetically altered host cells that are capable of secreting increased amounts of antibody will also provide a valuable method for creating cell hosts for product development. The invention described herein is directed to the creation of genetically altered cell hosts with increased antibody production via the blockade of MMR.

The invention facilitates the generation of high affinity antibodies and the production of cell lines with elevated levels of antibody production. Other advantages of the present invention are described in the examples and figures described herein.

SUMMARY OF THE INVENTION

The invention provides methods for generating genetically altered antibodies (including single chain molecules) and antibody producing cell hosts *in vitro* and *in vivo*, whereby the antibody possess a desired biochemical property(s), such as, but not limited to, increased antigen binding, increased gene expression, and/or enhanced extracellular secretion by the cell host. One method for identifying antibodies with increased binding activity or cells with increased antibody production is through the screening of MMR defective antibody producing cell clones that produce molecules with enhanced binding properties or clones that have been genetically altered to produce enhanced amounts of antibody product.

The antibody producing cells suitable for use in the invention include, but are not limited to rodent, primate, or human hybridomas or lymphoblastoids; mammalian cells transfected and expressing exogenous Ig subunits or chimeric single chain molecules; plant cells, yeast or bacteria transfected and expressing exogenous Ig subunits or chimeric single chain molecules.

Thus, the invention provides methods for making hypermutable antibody-producing cells by introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into cells that are capable of producing antibodies. The cells that are capable of producing antibodies include cells that naturally produce antibodies, and cells that are engineered to produce antibodies through the introduction of immunoglobulin encoding sequences. Conveniently, the introduction of polynucleotide sequences into cells is accomplished by transfection.

The invention also provides methods of making hypermutable antibody producing cells by introducing a dominant negative mismatch repair (MMR) gene such as *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2* into cells that are capable of producing antibodies. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134,

or a thymidine at nucleotide 424 of wild-type *PMS2*). The invention also provides methods in which mismatch repair gene activity is suppressed. This may be accomplished, for example, using antisense molecules directed against the mismatch repair gene or transcripts.

Other embodiments of the invention provide methods for making a hypermutable antibody producing cells by introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into fertilized eggs of animals. These methods may also include subsequently implanting the eggs into pseudo-pregnant females whereby the fertilized eggs develop into a mature transgenic animal. The mismatch repair genes may include, for example, *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2*. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*).

The invention further provides homogeneous compositions of cultured, hypermutable, mammalian cells that are capable of producing antibodies and contain a dominant negative allele of a mismatch repair gene. The mismatch repair genes may include, for example, *PMS2* (preferably human *PMS2*), *MLH1*, *PMS1*, *MSH2*, or *MSH2*. The dominant negative allele of a mismatch repair gene may be a truncation mutation of a mismatch repair gene (preferably a truncation mutation at codon 134, or a thymidine at nucleotide 424 of wild-type *PMS2*). The cells of the culture may contain *PMS2*, (preferably human *PMS2*), *MLH1*, or *PMS1*; or express a human *mutL* homolog, or the first 133 amino acids of hPMS2.

The invention further provides methods for generating a mutation in an immunoglobulin gene of interest by culturing an immunoglobulin producing cell selected for an immunoglobulin of interest wherein the cell contains a dominant negative allele of a mismatch repair gene. The properties of the immunoglobulin produced from the cells can be assayed to ascertain whether the immunoglobulin gene harbors a mutation. The assay may be directed to analyzing a polynucleotide encoding the immunoglobulin, or may be directed to the immunoglobulin polypeptide itself.

The invention also provides methods for generating a mutation in a gene affecting antibody production in an antibody-producing cell by culturing the cell expressing a dominant negative allele of a mismatch repair gene, and testing the cell to determine whether the cell

harbors mutations within the gene of interest, such that a new biochemical feature (*e.g.*, over-expression and/or secretion of immunoglobulin products) is generated. The testing may include analysis of the steady state expression of the immunoglobulin gene of interest, and/or analysis of the amount of secreted protein encoded by the immunoglobulin gene of interest. The invention also embraces prokaryotic and eukaryotic transgenic cells made by this process, including cells from rodents, non-human primates and humans.

Other aspects of the invention encompass methods of reversibly altering the hypermutability of an antibody producing cell, in which an inducible vector containing a dominant negative allele of a mismatch repair gene operably linked to an inducible promoter is introduced into an antibody-producing cell. The cell is treated with an inducing agent to express the dominant negative mismatch repair gene (which can be *PMS2* (preferably human *PMS2*), *MLH1*, or *PMS1*). Alternatively, the cell may be induced to express a human *mutL* homolog or the first 133 amino acids of hPMS2. In another embodiment, the cells may be rendered capable of producing antibodies by co-transfecting a preselected immunoglobulin gene of interest. The immunoglobulin genes of the hypermutable cells, or the proteins produced by these methods may be analyzed for desired properties, and induction may be stopped such that the genetic stability of the host cell is restored.

The invention also embraces methods of producing genetically altered antibodies by transfecting a polynucleotide encoding an immunoglobulin protein into a cell containing a dominant negative mismatch repair gene (either naturally or in which the dominant negative mismatch repair gene was introduced into the cell), culturing the cell to allow the immunoglobulin gene to become mutated and produce a mutant immunoglobulin, screening for a desirable property of said mutant immunoglobulin protein, isolating the polynucleotide molecule encoding the selected mutant immunoglobulin possessing the desired property, and transfecting said mutant polynucleotide into a genetically stable cell, such that the mutant antibody is consistently produced without further genetic alteration. The dominant negative mismatch repair gene may be *PMS2* (preferably human *PMS2*), *MLH1*, or *PMS1*. Alternatively, the cell may express a human *mutL* homolog or the first 133 amino acids of hPMS2.

The invention further provides methods for generating genetically altered cell lines that express enhanced amounts of an antigen binding polypeptide. These antigen-binding polyeptides may be, for example, immunoglobulins. The methods of the invention also include methods for generating genetically altered cell lines that secrete enhanced amounts of an antigen binding polypeptide. The cell lines are rendered hypermutable by dominant negative mismatch repair genes that provide an enhanced rate of genetic hypermutation in a cell producing antigen-binding polypeptides such as antibodies. Such cells include, but are not limited to hybridomas. Expression of enhanced amounts of antigen binding polypeptides may be through enhanced transcription or translation of the polynucleotides encoding the antigen binding polypeptides, or through the enhanced secretion of the antigen binding polypeptides, for example.

Methods are also provided for creating genetically altered antibodies *in vivo* by blocking the MMR activity of the cell host, or by transfecting genes encoding for immunoglobulin in a MMR defective cell host.

Antibodies with increased binding properties to an antigen due to genetic changes within the variable domain are provided in methods of the invention that block endogenous MMR of the cell host. Antibodies with increased binding properties to an antigen due to genetic changes within the CDR regions within the light and/or heavy chains are also provided in methods of the invention that block endogenous MMR of the cell host.

The invention provides methods of creating genetically altered antibodies in MMR defective Ab producer cell lines with enhanced pharmacokinetic properties in host organisms including but not limited to rodents, primates, and man.

These and other aspects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention, a method for making an antibody producing cell line hypermutable is provided. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into an antibody-producing cell. The cell becomes hypermutable as a result of the introduction of the gene.

In another embodiment of the invention, a method is provided for introducing a mutation into an endogenous gene encoding for an immunoglobulin polypeptide or a single chain antibody. A polynucleotide encoding a dominant negative allele of a MMR gene is

introduced into a cell. The cell becomes hypermutable as a result of the introduction and expression of the MMR gene allele. The cell further comprises an immunoglobulin gene of interest. The cell is grown and tested to determine whether the gene encoding for an immunoglobulin or a single chain antibody of interest harbors a mutation. In another aspect of the invention, the gene encoding the mutated immunoglobulin polypeptide or single chain antibody may be isolated and expressed in a genetically stable cell. In a preferred embodiment, the mutated antibody is screened for at least one desirable property such as, but not limited to, enhanced binding characteristics.

In another embodiment of the invention, a gene or set of genes encoding for Ig light and heavy chains or a combination therein are introduced into a mammalian cell host that is MMR defective. The cell is grown, and clones are analyzed for antibodies with enhanced binding characteristics.

In another embodiment of the invention, a method will be provided for producing new phenotypes of a cell. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction of the gene. The cell is grown. The cell is tested for the expression of new phenotypes where the phenotype is enhanced secretion of a polypeptide.

These and other embodiments of the invention provide the art with methods that can generate enhanced mutability in cells and animals as well as providing cells and animals harboring potentially useful mutations for the large-scale production of high affinity antibodies with beneficial pharmacokinetic profiles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Hybridoma cells stably expressing PMS2 and PMS134 MMR genes. Shown is steady state mRNA expression of MMR genes transfected into a murine hybridoma cell line. Stable expression was found after 3 months of continuous growth. The (–) lanes represent negative controls where no reverse transcriptase was added, and the (+) lanes represent samples reverse transcribed and PCR amplified for the MMR genes and an internal housekeeping gene as a control.

Figure 2. Creation of genetically hypermutable hybridoma cells. Dominant negative

MMR gene alleles were expressed in cells expressing a MMR-sensitive reporter gene. Dominant negative alleles such as PMS134 and the expression of MMR genes from other species results in antibody producer cells with a hypermutable phenotype that can be used to produce genetically altered immunoglobulin genes with enhanced biochemical features as well as lines with increased Ig expression and/or secretion. Values shown represent the amount of converted CPRG substrate which is reflective of the amount of function □-galactosidase contained within the cell from genetic alterations within the pCAR-OF reporter gene. Higher amounts of β-galactosidase activity reflect a higher mutation rate due to defective MMR.

Figure 3. Screening method for identifying antibody-producing cells containing antibodies with increased binding activity and/or increased expression/secretion

Figure 4. Generation of a genetically altered antibody with an increased binding activity. Shown are ELISA values from 96-well plates, screened for antibodies specific to hIgE. Two clones with a high binding value were found in HB134 cultures.

Figure 5. Sequence alteration within variable chain of an antibody (a mutation within the light chain variable region in MMR-defective HB134 antibody producer cells). Arrows indicate the nucleotide at which a mutation occurred in a subset of cells from a clone derived from HB134 cells. Panel A: The change results in a Thr to Ser change within the light chain variable region. The coding sequence is in the antisense direction. Panel B: The change results in a Pro to His change within the light chain variable region.

Figure 6. Generation of MMR-defective clones with enhanced steady state Ig protein levels. A Western blot of heavy chain immunglobulins from HB134 clones with high levels of MAb (>500ngs/ml) within the conditioned medium shows that a subset of clones express higher steady state levels of immunoglobulins (Ig). The H36 cell line was used as a control to measure steady state levels in the parental strain. Lane 1: fibroblast cells (negative control); Lane 2: H36 cell; Lane 3: HB134 clone with elevated MAb levels; Lane 4: HB134 clone with elevated MAb levels; Lane 5: HB134 clone with elevated MAb levels.

Methods have been discovered for developing hypermutable antibody-producing cells by taking advantage of the conserved mismatch repair (MMR) process of host cells. Dominant negative alleles of such genes, when introduced into cells or transgenic animals,

increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable. Hypermutable cells or animals can then be utilized to develop new mutations in a gene of interest. Blocking MMR in antibody-producing cells such as but not limited to: hybridomas; mammalian cells transfected with genes encoding for Ig light and heavy chains; mammalian cells transfected with genes encoding for single chain antibodies; eukaryotic cells transfected with Ig genes, can enhance the rate of mutation within these cells leading to clones that have enhanced antibody production and/or cells containing genetically altered antibodies with enhanced biochemical properties such as increased antigen binding. The process of MMR, also called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells. A MMR gene is a gene that encodes for one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a MMR complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base on the newer DNA strand is excised, and the excised base is replaced with the appropriate base, which is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication.

Dominant negative alleles cause a MMR defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a MMR gene is the human gene *hPMS2-134*, which carries a truncating mutation at codon 134 (SEQ ID NO:15). The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations, which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any allele which produces such effect can be used in this invention. Dominant negative alleles of a MMR gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms. Such alleles can be identified by screening cells for defective MMR activity. Cells from animals or humans with cancer can be screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic

DNA, cDNA, or mRNA from any cell encoding a MMR protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a MMR gene can also be created artificially, for example, by producing variants of the *hPMS2-134* allele or other MMR genes. Various techniques of site-directed mutagenesis can be used. The suitability of such alleles, whether natural or artificial, for use in generating hypermutable cells or animals can be evaluated by testing the mismatch repair activity caused by the allele in the presence of one or more wild-type alleles, to determine if it is a dominant negative allele.

A cell or an animal into which a dominant negative allele of a mismatch repair gene has been introduced will become hypermutable. This means that the spontaneous mutation rate of such cells or animals is elevated compared to cells or animals without such alleles. The degree of elevation of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or animal. The use of chemical mutagens such as but limited to methane sulfonate, dimethyl sulfonate, O6-methyl benzadine, MNU, ENU, etc. can be used in MMR defective cells to increase the rates an additional 10 to 100 fold that of the MMR deficiency itself.

According to one aspect of the invention, a polynucleotide encoding for a dominant negative form of a MMR protein is introduced into a cell. The gene can be any dominant negative allele encoding a protein, which is part of a MMR complex, for example, *PMS2, PMS1, MLH1*, or *MSH2*. The dominant negative allele can be naturally occurring or made in the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a chemically synthesized polynucleotide.

The polynucleotide can be cloned into an expression vector containing a constitutively active promoter segment (such as but not limited to CMV, SV40, Elongation Factor or LTR sequences) or to inducible promoter sequences such as the steroid inducible pIND vector (Invitrogen), where the expression of the dominant negative MMR gene can be regulated. The polynucleotide can be introduced into the cell by transfection.

According to another aspect of the invention, an immunoglobulin (Ig) gene, a set of Ig genes or a chimeric gene containing whole or parts of an Ig gene can be transfected into MMR deficient cell hosts, the cell is grown and screened for clones containing genetically altered Ig genes with new biochemical features. MMR defective cells may be of human,

primates, mammals, rodent, plant, yeast or of the prokaryotic kingdom. The mutated gene encoding the Ig with new biochemical features may be isolated from the respective clones and introduced into genetically stable cells (*i.e.*, cells with normal MMR) to provide clones that consistently produce Ig with the new biochemical features. The method of isolating the Ig gene encoding Ig with new biochemical features may be any method known in the art. Introduction of the isolated polynucleotide encoding the Ig with new biochemical features may also be performed using any method known in the art, including, but not limited to transfection of an expression vector containing the polynucleotide encoding the Ig with new biochemical features. As an alternative to transfecting an Ig gene, a set of Ig genes or a chimeric gene containing whole or parts of an Ig gene into an MMR deficient host cell, such Ig genes may be transfected simultaneously with a gene encoding a dominant negative mismatch repair gene into a genetically stable cell to render the cell hypermutable.

Transfection is any process whereby a polynucleotide is introduced into a cell. The process of transfection can be carried out in a living animal, *e.g.*, using a vector for gene therapy, or it can be carried out *in vitro*, *e.g.*, using a suspension of one or more isolated cells in culture. The cell can be any type of eukaryotic cell, including, for example, cells isolated from humans or other primates, mammals or other vertebrates, invertebrates, and single celled organisms such as protozoa, yeast, or bacteria.

In general, transfection will be carried out using a suspension of cells, or a single cell, but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the MMR gene, the cell can be grown and reproduced in culture. If the transfection is stable, such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

An isolated cell is a cell obtained from a tissue of humans or animals by mechanically separating out individual cells and transferring them to a suitable cell culture medium, either

with or without pretreatment of the tissue with enzymes, *e.g.*, collagenase or trypsin. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a eukaryotic organism in the form of a primary cell culture or an immortalized cell line, or may be derived from suspensions of single-celled organisms.

A polynucleotide encoding for a dominant negative form of a MMR protein can be introduced into the genome of an animal by producing a transgenic animal. The animal can be any species for which suitable techniques are available to produce transgenic animals. For example, transgenic animals can be prepared from domestic livestock, *e.g.*, bovine, swine, sheep, goats, horses, etc.; from animals used for the production of recombinant proteins, *e.g.*, bovine, swine, or goats that express a recombinant polypeptide in their milk; or experimental animals for research or product testing, *e.g.*, mice, rats, guinea pigs, hamsters, rabbits, etc. Cell lines that are determined to be MMR defective can then be used as a source for producing genetically altered immunoglobulin genes *in vitro* by introducing whole, intact immunoglobulin genes and/or chimeric genes encoding for single chain antibodies into MMR defective cells from any tissue of the MMR defective animal.

Once a transfected cell line or a colony of transgenic animals has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest can be any gene naturally possessed by the cell line or transgenic animal or introduced into the cell line or transgenic animal. An advantage of using such cells or animals to induce mutations is that the cell or animal need not be exposed to mutagenic chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers. However, chemical mutagens may be used in combination with MMR deficiency, which renders such mutagens less toxic due to an undetermined mechanism. Hypermutable animals can then be bred and selected for those producing genetically variable B-cells that may be isolated and cloned to identify new cell lines that are useful for producing genetically variable cells. Once a new trait is identified, the dominant negative MMR gene allele can be removed by directly knocking out the allele by technologies used by those skilled in the art or by breeding to mates lacking the dominant negative allele to select for offspring with a desired trait and a stable genome. Another alternative is to use a CRE-LOX expression

system, whereby the dominant negative allele is spliced from the animal genome once an animal containing a genetically diverse immunoglobulin profile has been established. Yet another alternative is the use of inducible vectors such as the steroid induced pIND (Invitrogen) or pMAM (Clonetech) vectors which express exogenous genes in the presence of corticosteroids.

Mutations can be detected by analyzing for alterations in the genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by screening for the production of antibody titers. A mutant polypeptide can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen for alteration of any property of the cell or animal associated with the function of the gene of interest, such as but not limited to Ig secretion.

Examples of mismatch repair proteins and nucleic acid sequences include the following:

PMS2 (mouse) (SEQ ID NO:5)

```
MEQTEGVSTE CAKAIKPIDG KSVHQICSGQ VILSLSTAVK ELIENSVDAG ATTIDLRLKD  60
YGVDLIEVSD NGCGVEEENF EGLALKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
TISTCHGSAS VGTRLVFDHN GKITQKTPYP RPKGTTVSVQ HLFYTLPVRY KEFQRNIKKE 180
YSKMVQVLQA YCIISAGVRV SCTNQLGQGK RHAVVCTSGT SGMKENIGSV FGQKQLQSLI 240
PFVQLPPSDA VCEEYGLSTS GRHKTFSTFR ASFHSARTAP GGVQQTGSFS SSIRGPVTQQ 300
RSLSLSMRFY HMYNRHQYPF VVLNVSVDSE CVDINVTPDK RQILLQEEKL LLAVLKTSLI 360
GMFDSDANKL NVNQQPLLDV EGNLVKLHTA ELEKPVPGKQ DNSPSLKSTA DEKRVASISR 420
LREAFSLHPT KEIKSRGPET AELTRSFPSE KRGVLSSYPS DVISYRGLRG SQDKLVSPTD 480
SPGDCMDREK IEKDSGLSST SAGSEEEFST PEVASSFSSD YNVSSLEDRP SQETINCGDL 540
DCRPPGTGQS LKPEDHGYQC KALPLARLSP TNAKRFKTEE RPSNVNISQR LPGPQSTSAA 600
EVDVAIKMNK RIVLLEFSLS SLAKRMKQLQ HLKAQNKHEL SYRKFRAKIC PGENQAAEDE 660
LRKEISKSMF AEMEILGQFN LGFIVTKLKE DLFLVDQHAA DEKYNFEMLQ QHTVLQAQRL 720
ITPQTLNLTA VNEAVLIENL EIFRKNGFDF VIDEDAPVTE RAKLISLPTS KNWTFGPQDI 780
DELIFMLSDS PGVMCRPSRV RQMFASRACR KSVMIGTALN ASEMKKLITH MGEMDHPWNC 840
PHGRPTMRHV ANLDVISQN                                             859
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PMS2 (mouse cDNA) (SEQ ID NO:6)

```
gaattccggt gaaggtcctg aagaatttcc agattcctga gtatcattgg aggagacaga 60
taacctgtcg tcaggtaacg atggtgtata tgcaacagaa atgggtgttc ctggagacgc 120
gtcttttccc gagagcggca ccgcaactct cccgcggtga ctgtgactgg aggagtcctg 180
catccatgga gcaaaccgaa ggcgtgagta cagaatgtgc taaggccatc aagcctattg 240
atgggaagtc agtccatcaa atttgttctg ggcaggtgat actcagttta agcaccgctg 300
tgaaggagtt gatagaaaat agtgtagatg ctggtgctac tactattgat ctaaggctta 360
aagactatgg ggtggacctc attgaagttt cagacaatgg atgtggggta gaagaagaaa 420
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actttgaagg tctagctctg aaacatcaca catctaagat tcaagagttt gccgacctca 480
cgcaggttga aactttcggc tttcgggggg aagctctgag ctctctgtgt gcactaagtg 540
atgtcactat atctacctgc cacgggtctg caagcgttgg gactcgactg gtgtttgacc 600
ataatgggaa aatcacccag aaaactccct acccccgacc taaaggaacc acagtcagtg 660
tgcagcactt attttataca ctacccgtgc gttacaaaga gtttcagagg aacattaaaa 720
aggagtattc caaaatggtg caggtcttac aggcgtactg tatcatctca gcaggcgtcc 780
gtgtaagctg cactaatcag ctcggacagg ggaagcggca cgctgtggtg tgcacaagcg 840
gcacgtctgg catgaaggaa aatatcgggt ctgtgtttgg ccagaagcag ttgcaaagcc 900
tcattccttt tgttcagctg ccccctagtg acgctgtgtg tgaagagtac ggcctgagca 960
cttcaggacg ccacaaaacc ttttctacgt ttcgggcttc atttcacagt gcacgcacgg 1020
cgccgggagg agtgcaacag acaggcagtt tttcttcatc aatcagaggc cctgtgaccc 1080
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catttgtcgt ccttaacgtt tccgttgact cagaatgtgt ggatattaat gtaactccag 1200
ataaaaggca aattctacta caagaagaga agctattgct ggccgtttta aagacctcct 1260
tgataggaat gtttgacagt gatgcaaaca agcttaatgt caaccagcag ccactgctag 1320
atgttgaagg taacttagta aagctgcata ctgcagaact agaaaagcct gtgccaggaa 1380
agcaagataa ctctccttca ctgaagagca cagcagacga gaaaagggta gcatccatct 1440
ccaggctgag agaggccttt tctcttcatc ctactaaaga gatcaagtct aggggtccag 1500
agactgctga actgacacgg agttttccaa gtgagaaaag gggcgtgtta tcctcttatc 1560
cttcagacgt catctcttac agaggcctcc gtggctcgca ggacaaattg gtgagtccca 1620
cggacagccc tggtgactgt atggacagag agaaaataga aaaagactca gggctcagca 1680
gcacctcagc tggctctgag gaagagttca gcaccccaga agtggccagt agctttagca 1740
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acctggactg ccgtcctcca ggtacaggac agtccttgaa gccagaagac catggatatc 1860
aatgcaaagc tctacctcta gctcgtctgt cacccacaaa tgccaagcgc ttcaagacag 1920
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cagctgaggt cgatgtagcc ataaaaatga ataagagaat cgtgctcctc gagttctctc 2040
tgagttctct agctaagcga atgaagcagt tacagcacct aaaggcgcag aacaaacatg 2100
aactgagtta cagaaaattt agggccaaga tttgccctgg agaaaaccaa gcagcagaag 2160
atgaactcag aaaagagatt agtaaatcga tgtttgcaga gatggagatc ttgggtcagt 2220
ttaacctggg atttatagta accaaactga aagaggacct cttcctggtg gaccagcatg 2280
ctgcggatga gaagtacaac tttgagatgc tgcagcagca cacggtgctc caggcgcaga 2340
ggctcatcac accccagact ctgaacttaa ctgctgtcaa tgaagctgta ctgatagaaa 2400
atctggaaat attcagaaag aatggctttg actttgtcat tgatgaggat gctccagtca 2460
ctgaaagggc taaattgatt tccttaccaa ctagtaaaaa ctggaccttt ggaccccaag 2520
atatagatga actgatcttt atgttaagtg acagccctgg ggtcatgtgc cggccctcac 2580
gagtcagaca gatgtttgct tccagagcct gtcggaagtc agtgatgatt ggaacggcgc 2640
tcaatgcgag cgagatgaag aagctcatca cccacatggg tgagatggac caccctgga 2700
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actgacacac cccttgtagc atagagttta ttacagattg ttcggtttgc aaagagaagg 2820
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agactcaatt caaggacaaa aaaaaaaaga tatttttgaa gccttttaaa aaaaaa     3056
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PMS2 (human) (SEQ ID NO:7)

```
MERAESSSTE PAKAIKPIDR KSVHQICSGQ VVLSLSTAVK ELVENSLDAG ATNIDLKLKD 60
YGVDLIEVSD NGCGVEEENF EGLTLKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
TISTCHASAK VGTRLMFDHN GKIIQKTPYP RPRGTTVSVQ QLFSTLPVRH KEFQRNIKKE 180
YAKMVQVLHA YCIISAGIRV SCTNQLGQGK RQPVVCTGGS PSIKENIGSV FGQKQLQSLI 240
PFVQLPPSDS VCEEYGLSCS DALHNLFYIS GFISQCTHGV GRSSTDRQFF FINRRPCDPA 300
KVCRLVNEVY HMYNRHQYPF VVLNISVDSE CVDINVTPDK RQILLQEEKL LLAVLKTSLI 360
GMFDSDVNKL NVSQQPLLDV EGNLIKMHAA DLEKPMVEKQ DQSPSLRTGE EKKDVSISRL 420
REAFSLRHTT ENKPHSPKTP EPRRSPLGQK RGMLSSSTSG AISDKGVLRP QKEAVSSSHG 480
PSDPTDRAEV EKDSGHGSTS VDSEGFSIPD TGSHCSSEYA ASSPGDRGSQ EHVDSQEKAP 540
ETDDSFSDVD CHSNQEDTGC KFRVLPQPTN LATPNTKRFK KEEILSSSDI CQKLVNTQDM 600
SASQVDVAVK INKKVVPLDF SMSSLAKRIK QLHHEAQQSE GEQNYRKFRA KICPGENQAA 660
EDELRKEISK TMFAEMEIIG QFNLGFIITK LNEDIFIVDQ HATDEKYNFE MLQQHTVLQG 720
QRLIAPQTLN LTAVNEAVLI ENLEIFRKNG FDFVIDENAP VTERAKLISL PTSKNWTFGP 780
QDVDELIFML SDSPGVMCRP SRVKQMFASR ACRKSVMIGT ALNTSEMKKL ITHMGEMDHP 840
WNCPHGRPTM RHIANLGVIS QN                                        862
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PMS2 (human cDNA) (SEQ ID NO:8)

```
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aaggccatca aacctattga tcggaagtca gtccatcaga tttgctctgg gcaggtggta 120
ctgagtctaa gcactgcggt aaaggagtta gtagaaaaca gtctggatgc tggtgccact 180
aatattgatc taaagcttaa ggactatgga gtggatctta ttgaagtttc agacaatgga 240
tgtggggtag aagaagaaaa cttcgaaggc ttaactctga aacatcacac atctaagatt 300
caagagtttg ccgacctaac tcaggttgaa acttttggct ttcgggggga agctctgagc 360
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atcatttcag caggcatccg tgtaagttgc accaatcagc ttggacaagg aaaacgacag 660
cctgtggtat gcacaggtgg aagccccagc ataaaggaaa atatcggctc tgtgtttggg 720
cagaagcagt tgcaaagcct cattcctttt gttcagctgc cccctagtga ctccgtgtgt 780
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gaggatatct tcatagtgga ccagcatgcc acggacgaga agtataactt cgagatgctg 2160
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tttatcgcag atttttatgt tttgaaagac agagtcttca ctaacctttt ttgttttaaa 2700
atgaaacctg ctacttaaaa aaaatacaca tcacacccat ttaaaagtga tcttgagaac 2760
cttttcaaac c                                                      2771
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PMS1 (human) (SEQ ID NO:9)

```
MKQLPAATVR LLSSSQIITS VVSVVKELIE NSLDAGATSV DVKLENYGFD KIEVRDNGEG  60
IKAVDAPVMA MKYYTSKINS HEDLENLTTY GFRGEALGSI CCIAEVLITT RTAADNFSTQ 120
YVLDGSGHIL SQKPSHLGQG TTVTALRLFK NLPVRKQFYS TAKKCKDEIK KIQDLLMSFG 180
ILKPDLRIVF VHNKAVIWQK SRVSDHKMAL MSVLGTAVMN NMESFQYHSE ESQIYLSGFL 240
PKCDADHSFT SLSTPERSFI FINSRPVHQK DILKLIRHHY NLKCLKESTR LYPVFFLKID 300
VPTADVDVNL TPDKSQVLLQ NKESVLIALE NLMTTCYGPL PSTNSYENNK TDVSAADIVL 360
SKTAETDVLF NKVESSGKNY SNVDTSVIPF QNDMHNDESG KNTDDCLNHQ ISIGDFGYGH 420
CSSEISNIDK NTKNAFQDIS MSNVSWENSQ TEYSKTCFIS SVKHTQSENG NKDHIDESGE 480
NEEEAGLENS SEISADEWSR GNILKNSVGE NIEPVKILVP EKSLPCKVSN NNYPIPEQMN 540
LNEDSCNKKS NVIDNKSGKV TAYDLLSNRV IKKPMSASAL FVQDHRPQFL IENPKTSLED 600
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```
ATLQIEELWK TLSEEEKLKY EEKATKDLER YNSQMKRAIE QESQMSLKDG RKKIKPTSAW 660
NLAQKHKLKT SLSNQPKLDE LLQSQIEKRR SQNIKMVQIP FSMKNLKINF KKQNKVDLEE 720
KDEPCLIHNL RFPDAWLMTS KTEVMLLNPY RVEEALLFKR LLENHKLPAE PLEKPIMLTE 780
SLFNGSHYLD VLYKMTADDQ RYSGSTYLSD PRLTANGFKI KLIPGVSITE NYLEIEGMAN 840
CLPFYGVADL KEILNAILNR NAKEVYECRP RKVISYLEGE AVRLSRQLPM YLSKEDIQDI 900
IYRMKHQFGN EIKECVHGRP FFHHLTYLPE TT                              932
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PMS1 (human) (SEQ ID NO:10)

```
ggcacgagtg gctgcttgcg gctagtggat ggtaattgcc tgcctcgcgc tagcagcaag 60
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gttctcagat catcacttcg gtggtcagtg ttgtaaaaga gcttattgaa aactccttgg 180
atgctggtgc cacaagcgta gatgttaaac tggagaacta tggatttgat aaaattgagg 240
tgcgagataa cggggagggt atcaaggctg ttgatgcacc tgtaatggca atgaagtact 300
acacctcaaa aataaatagt catgaagatc ttgaaaattt gacaacttac ggttttcgtg 360
gagaagcctt ggggtcaatt tgttgtatag ctgaggtttt aattacaaca agaacggctg 420
ctgataattt tagcacccag tatgttttag atggcagtgg ccacatactt tctcagaaac 480
cttcacatct tggtcaaggt acaactgtaa ctgctttaag attatttaag aatctacctg 540
taagaaagca gttttactca actgcaaaaa aatgtaaaga tgaaataaaa aagatccaag 600
atctcctcat gagctttggt atccttaaac ctgacttaag gattgtcttt gtacataaca 660
aggcagttat ttggcagaaa agcagagtat cagatcacaa gatggctctc atgtcagttc 720
tggggactgc tgttatgaac aatatggaat cctttcagta ccactctgaa gaatctcaga 780
tttatctcag tggatttctt ccaaagtgtg atgcagacca ctctttcact agtctttcaa 840
caccagaaag aagtttcatc ttcataaaca gtcgaccagt acatcaaaaa gatatcttaa 900
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cgacttgtta tggaccatta cctagtacaa attcttatga aaataataaa acagatgttt 1140
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aatcatctgg aaagaattat tcaaatgttg atacttcagt cattccattc caaaatgata 1260
tgcataatga tgaatctgga aaaaacactg atgattgttt aaatcaccag ataagtattg 1320
gtgactttgg ttatggtcat tgtagtagtg aaatttctaa cattgataaa aacactaaga 1380
atgcatttca ggacatttca atgagtaatg tatcatggga gaactctcag acggaatata 1440
gtaaaacttg ttttataagt tccgttaagc acacccagtc agaaaatggc aataaagacc 1500
atatagatga gagtggggaa aatgaggaag aagcaggtct tgaaaactct tcggaaattt 1560
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ttaaaatggt acagatcccc ttttctatga aaaacttaaa aataaatttt aagaaacaaa 2220
acaaagttga cttagaagag aaggatgaac cttgcttgat ccacaatctc aggtttcctg 2280
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aagccctgct atttaaaaga cttcttgaga atcataaact tcctgcagag ccactggaaa 2400
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ttaatgctat attaaacaga aatgcaaagg aagtttatga atgtagacct cgcaaagtga 2700
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taaatatgtt taagaagatt agttaccatt gaaattggtt ctgtcataaa acagcatgag 2940
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aac                                                              3063
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MSH2 (human) (SEQ ID NO:11)

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MAVQPKETLQ LESAAEVGFV RFFQGMPEKP TTTVRLFDRG DFYTAHGEDA LLAAREVFKT 60
QGVIKYMGPA GAKNLQSVVL SKMNFESFVK DLLLVRQYRV EVYKNRAGNK ASKENDWYLA 120
YKASPGNLSQ FEDILFGNND MSASIGVVGV KMSAVDGQRQ VGVGYVDSIQ RKLGLCEFPD 180
NDQFSNLEAL LIQIGPKECV LPGGETAGDM GKLRQIIQRG GILITERKKA DFSTKDIYQD 240
LNRLLKGKKG EQMNSAVLPE MENQVAVSSL SAVIKFLELL SDDSNFGQFE LTTFDFSQYM 300
KLDIAAVRAL NLFQGSVEDT TGSQSLAALL NKCKTPQGQR LVNQWIKQPL MDKNRIEERL 360
NLVEAFVEDA ELRQTLQEDL LRRFPDLNRL AKKFQRQAAN LQDCYRLYQG INQLPNVIQA 420
LEKHEGKHQK LLLAVFVTPL TDLRSDFSKF QEMIETTLDM DQVENHEFLV KPSFDPNLSE 480
LREIMNDLEK KMQSTLISAA RDLGLDPGKQ IKLDSSAQFG YYFRVTCKEE KVLRNNKNFS 540
TVDIQKNGVK FTNSKLTSLN EEYTKNKTEY EEAQDAIVKE IVNISSGYVE PMQTLNDVLA 600
QLDAVVSFAH VSNGAPVPYV RPAILEKGQG RIILKASRHA CVEVQDEIAF IPNDVYFEKD 660
KQMFHIITGP NMGGKSTYIR QTGVIVLMAQ IGCFVPCESA EVSIVDCILA RVGAGDSQLK 720
GVSTFMAEML ETASILRSAT KDSLIIIDEL GRGTSTYDGF GLAWAISEYI ATKIGAFCMF 780
ATHFHELTAL ANQIPTVNNL HVTALTTEET LTMLYQVKKG VCDQSFGIHV AELANFPKHV 840
IECAKQKALE LEEFQYIGES QGYDIMEPAA KKCYLEREQG EKIIQEFLSK VKQMPFTEMS 900
EENITIKLKQ LKAEVIAKNN SFVNEIISRI KVTT                           934
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MSH2 (human cDNA) (SEQ ID NO:12)

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ggcgggaaac agcttagtgg gtgtggggtc gcgcattttc ttcaaccagg aggtgaggag 60
gtttcgacat ggcggtgcag ccgaaggaga cgctgcagtt ggagagcgcg gccgaggtcg 120
gcttcgtgcg cttctttcag ggcatgccgg agaagccgac caccacagtg cgccttttcg 180
accggggcga cttctatacg gcgcacggcg aggacgcgct gctggccgcc cgggaggtgt 240
tcaagaccca gggggtgatc aagtacatgg ggccggcagg agcaaagaat ctgcagagtg 300
ttgtgcttag taaaatgaat tttgaatctt ttgtaaaaga tcttcttctg gttcgtcagt 360
atagagttga agtttataag aatagagctg gaaataaggc atccaaggag aatgattggt 420
atttggcata taaggcttct cctggcaatc tctctcagtt tgaagacatt ctctttggta 480
acaatgatat gtcagcttcc attggtgttg tgggtgttaa aatgtccgca gttgatggcc 540
agagacaggt tggagttggg tatgtggatt ccatacagag gaaactagga ctgtgtgaat 600
tccctgataa tgatcagttc tccaatcttg aggctctcct catccagatt ggaccaaagg 660
aatgtgtttt acccggagga gagactgctg gagacatggg gaaactgaga cagataattc 720
aaagaggagg aattctgatc acagaaagaa aaaaagctga ctttccaca aaagacattt 780
atcaggacct caaccggttg ttgaaaggca aaaagggaga gcagatgaat agtgctgtat 840
tgccagaaat ggagaatcag gttgcagttt catcactgtc tgcggtaatc aagtttttag 900
aactcttatc agatgattcc aactttggac agtttgaact gactactttt gacttcagcc 960
agtatatgaa attggatatt gcagcagtca gagcccttaa ccttttcag ggttctgttg 1020
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gacaaagact tgttaaccag tggattaagc agcctctcat ggataagaac agaatagagg 1140
agagattgaa tttagtggaa gcttttgtag aagatgcaga attgaggcag actttacaag 1200
aagatttact tcgtcgattc ccagatctta accgacttgc caagaagttt caaagacaag 1260
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ctttaaatga agagtatacc aaaaataaaa cagaatatga agaagcccag gatgccattg 1800
ttaaagaaat tgtcaatatt tcttcaggct atgtagaacc aatgcagaca ctcaatgatg 1860
tgttagctca gctagatgct gttgtcagct ttgctcacgt gtcaaatgga gcacctgttc 1920
catatgtacg accagccatt ttggagaaag gacaaggaag aattatatta aaagcatcca 1980
ggcatgcttg tgttgaagtt caagatgaaa ttgcatttat tcctaatgac gtatactttg 2040
aaaaagataa acagatgttc cacatcatta ctggccccaa tatgggaggt aaatcaacat 2100
atattcgaca aactggggtg atagtactca tggcccaaat tgggtgtttt gtgccatgtg 2160
agtcagcaga agtgtccatt gtggactgca tcttagcccg agtaggggct ggtgacagtc 2220
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aattgaaagg agtctccacg ttcatggctg aaatgttgga aactgcttct atcctcaggt 2280
ctgcaaccaa agattcatta ataatcatag atgaattggg aagaggaact tctacctacg 2340
atggatttgg gttagcatgg gctatatcag aatacattgc aacaaagatt ggtgcttttt 2400
gcatgtttgc aacccatttt catgaactta ctgccttggc caatcagata ccaactgtta 2460
ataatctaca tgtcacagca ctcaccactg aagagacctt aactatgctt tatcaggtga 2520
agaaaggtgt ctgtgatcaa agttttggga ttcatgttgc agagcttgct aatttcccta 2580
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gagaatcgca aggatatgat atcatggaac cagcagcaaa gaagtgctat ctggaaagag 2700
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agaataatag ctttgtaaat gaaatcattt cacgaataaa agttactacg tgaaaaatcc 2880
cagtaatgga atgaaggtaa tattgataag ctattgtctg taatagtttt atattgtttt 2940
atattaaccc tttttccata gtgttaactg tcagtgccca tgggctatca acttaataag 3000
atatttagta atattttact ttgaggacat tttcaaagat ttttattttg aaaaatgaga 3060
gctgtaactg aggactgttt gcaattgaca taggcaataa taagtgatgt gctgaatttt 3120
ataaataaaa tcatgtagtt tgtgg                                      3145
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MLH1 (human) (SEQ ID NO:13)

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MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ  60
IQDNGTGIRK EDLDIVCERF TTSKLQSFED LASISTYGFR GEALASISHV AHVTITTKTA 120
DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV 180
GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF 240
KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP 300
QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV 360
KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS 420
SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE 480
MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL 540
AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE 600
DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL 660
ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV 720
YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC                          756
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MLH1 (human) (SEQ ID NO:14)

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cttggctctt ctggcgccaa aatgtcgttc gtggcagggg ttattcggcg gctggacgag 60
acagtggtga accgcatcgc ggcgggggaa gttatccagc ggccagctaa tgctatcaaa 120
gagatgattg agaactgttt agatgcaaaa tccacaagta ttcaagtgat tgttaaagag 180
ggaggcctga agttgattca gatccaagac aatggcaccg ggatcaggaa agaagatctg 240
gatattgtat gtgaaaggtt cactactagt aaactgcagt cctttgagga tttagccagt 300
atttctacct atggctttcg aggtgaggct ttggccagca taagccatgt ggctcatgtt 360
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aaactgaaag cccctcctaa accatgtgct ggcaatcaag ggacccagat cacggtggag 480
gacctttttt acaacatagc cacgaggaga aaagctttaa aaaatccaag tgaagaatat 540
gggaaaattt tggaagttgt tggcaggtat tcagtacaca atgcaggcat tagtttctca 600
gttaaaaaac aaggagagac agtagctgat gttaggacac tacccaatgc ctcaaccgtg 660
gacaatattc gctccatctt tggaaatgct gttagtcgag aactgataga aattggatgt 720
gaggataaaa ccctagcctt caaaatgaat ggttacatat ccaatgcaaa ctactcagtg 780
aagaagtgca tcttcttact cttcatcaac catcgtctgg tagaatcaac ttccttgaga 840
aaagccatag aaacagtgta tgcagcctat ttgcccaaaa acacacaccc attcctgtac 900
ctcagtttag aaatcagtcc ccagaatgtg gatgttaatg tgcaccccac aaagcatgaa 960
gttcacttcc tgcacgagga gagcatcctg gagcgggtgc agcagcacat cgagagcaag 1020
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gatgcatttc tgcagcctct gagcaaaccc ctgtccagtc agccccaggc cattgtcaca 1260
gaggataaga cagatatttc tagtggcagg gctaggcagc aagatgagga gatgcttgaa 1320
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gggacttcag aaatgtcaga gaagagagga cctacttcca gcaaccccag aaagagacat 1440
cgggaagatt ctgatgtgga aatggtggaa gatgattccc gaaaggaaat gactgcagct 1500
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tgtacccccc ggagaaggat cattaacctc actagtgttt tgagtctcca ggaagaaatt 1560
aatgagcagg gacatgaggt tctccgggag atgttgcata accactcctt cgtgggctgt 1620
gtgaatcctc agtgggcctt ggcacagcat caaaccaagt tataccttct caacaccacc 1680
aagcttagtg aagaactgtt ctaccagata ctcatttatg attttgccaa ttttggtgtt 1740
ctcaggttat cggagccagc accgctcttt gaccttgcca tgcttgcctt agatagtcca 1800
gagagtggct ggacagagga agatggtccc aaagaaggac ttgctgaata cattgttgag 1860
tttctgaaga agaaggctga gatgcttgca gactatttct ctttggaaat tgatgaggaa 1920
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cctatcttca ttcttcgact agccactgag gtgaattggg acgaagaaaa ggaatgtttt 2040
gaaagcctca gtaaagaatg cgctatgttc tattccatcc ggaagcagta catatctgag 2100
gagtcgaccc tctcaggcca gcagagtgaa gtgcctggct ccattccaaa ctcctggaag 2160
tggactgtgg aacacattgt ctataaagcc ttgcgctcac acattctgcc tcctaaacat 2220
ttcacagaag atggaaatat cctgcagctt gctaacctgc ctgatctata caaagtcttt 2280
gagaggtgtt aaatatggtt atttatgcac tgtgggatgt gttcttcttt ctctgtattc 2340
cgatacaaag tgttgtatca aagtgtgata tacaaagtgt accaacataa gtgttggtag 2400
cacttaagac ttatacttgc cttctgatag tattccttta tacacagtgg attgattata 2460
aataaataga tgtgtcttaa cata                                       2484
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hPMS2-134 (human) (SEQ ID NO:15)

```
MERAESSSTE PAKAIKPIDR KSVHQICSGQ VVLSLSTAVK ELVENSLDAG ATNIDLKLKD 60
YGVDLIEVSD NGCGVEEENF EGLTLKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
TISTCHASAK VGT                                                   133
```

hPMS2-134 (human cDNA) (SEQ ID NO:16)

```
cgaggcggat cgggtgttgc atccatggag cgagctgaga gctcgagtac agaacctgct 60
aaggccatca aacctattga tcggaagtca gtccatcaga tttgctctgg gcaggtggta 120
ctgagtctaa gcactgcggt aaaggagtta gtagaaaaca gtctggatgc tggtgccact 180
aatattgatc taaagcttaa ggactatgga gtggatctta ttgaagtttc agacaatgga 240
tgtggggtag aagaagaaaa cttcgaaggc ttaactctga aacatcacac atctaagatt 300
caagagtttg ccgacctaac tcaggttgaa acttttggct ttcgggggga agctctgagc 360
tcactttgtg cactgagcga tgtcaccatt tctacctgcc acgcatcggc gaaggttgga 420
acttga                                                           426
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For further information on the background of the invention the following references may be consulted, each of which is incorporated herein by reference in its entirety:

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The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1: Stable expression of dominant negative MMR genes in hybridoma cells

It has been previously shown by Nicolaides et al. (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641) that the expression of a dominant negative allele in an otherwise MMR proficient cell could render these host cells MMR deficient. The creation of MMR deficient cells can lead to the generation of genetic alterations throughout the entire genome of a host organisms offspring, yielding a population of genetically altered offspring or siblings that may produce biochemicals with altered properties. This patent application teaches of the use of dominant negative MMR genes in antibody-producing cells, including but not limited to rodent hybridomas, human hybridomas, chimeric rodent cells producing human immunoglobulin gene products, human cells expressing immunoglobulin genes, mammalian cells producing single chain antibodies, and prokaryotic cells producing mammalian immunoglobulin genes or chimeric immunoglobulin molecules such as those contained within single-chain antibodies. The cell expression systems described above that are used to produce antibodies are well known by those skilled in the art of antibody therapeutics.

To demonstrate the ability to create MMR defective hybridomas using dominant negative alleles of MMR genes, we first transfected a mouse hybridoma cell line that is known to produce and antibody directed against the human IgE protein with an expression vector containing the human PMS2 (cell line referred to as HBPMS2), the previously

published dominant negative PMS2 mutant referred herein as PMS134 (cell line referred to as HB134), or with no insert (cell line referred to as HBvec). The results showed that the PMS134 mutant could indeed exert a robust dominant negative effect, resulting in biochemical and genetic manifestations of MMR deficiency. Unexpectedly was the finding that the full length PMS2 also resulted in a lower MMR activity while no effect was seen in cells containing the empty vector. A brief description of the methods is provided below.

The MMR proficient mouse H36 hybridoma cell line was transfected with various *hPMS2* expression plasmids plus reporter constructs for assessing MMR activity. The MMR genes were cloned into the pEF expression vector, which contains the elongation factor promoter upstream of the cloning site followed by a mammalian polyadenylation signal. This vector also contains the NEOr gene that allows for selection of cells retaining this plasmid. Briefly, cells were transfected with 1 μg of each vector using polyliposomes following the manufacturer's protocol (Life Technologies). Cells were then selected in 0.5 mg/ml of G418 for 10 days and G418 resistant cells were pooled together to analyze for gene expression. The pEF construct contains an intron that separates the exon 1 of the EF gene from exon 2, which is juxtaposed to the 5' end of the polylinker cloning site. This allows for a rapid reverse transcriptase polymerase chain reaction (RT-PCR) screen for cells expressing the spliced products. At day 17, 100,000 cells were isolated and their RNA extracted using the trizol method as previously described (Nicolaides N.C., Kinzler, K.W., and Vogelstein, B. (1995) Analysis of the 5' region of PMS2 reveals heterogeneous transcripts and a novel overlapping gene. *Genomics* 29:329-334). RNAs were reverse transcribed using Superscript II (Life Technologies) and PCR amplified using a sense primer located in exon 1 of the EF gene (5'-ttt cgc aac ggg ttt gcc g-3') and an antisense primer (5'-gtt tca gag tta agc ctt cg-3') centered at nt 283 of the published human PMS2 cDNA, which will detect both the full length as well as the PMS134 gene expression. Reactions were carried out using buffers and conditions as previously described (Nicolaides, N.C., *et al.* (1995) Genomic organization of the human PMS2 gene family. *Genomics* 30:195-206), using the following amplification parameters: 94°C for 30 sec, 52°C for 2 min, 72°C for 2 min, for 30 cycles. Reactions were analyzed on agarose gels. Figure 1 shows a representative example of PMS expression in stably transduced H36 cells.

Expression of the protein encoded by these genes were confirmed via western blot using a polyclonal antibody directed to the first 20 amino acids located in the N-terminus of the protein following the procedures previously described (data not shown) (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641.

EXAMPLE 2: hPMS134 Causes a Defect in MMR Activity and hypermutability in hybridoma cells

A hallmark of MMR deficiency is the generation of unstable microsatellite repeats in the genome of host cells. This phenotype is referred to as microsatellite instability (MI) (Modrich, P. (1994) Mismatch repair, genetic stability, and cancer *Science* 266:1959-1960; Palombo, F., *et al.* (1994) Mismatch repair and cancer *Nature* 36:417). MI consists of deletions and/or insertions within repetitive mono-, di- and/or tri nucleotide repetitive sequences throughout the entire genome of a host cell. Extensive genetic analysis eukaryotic cells have found that the only biochemical defect that is capable of producing MI is defective MMR (Strand, M., *et al.* (1993) Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair *Nature* 365:274-276; Perucho, M. (1996) Cancer of the microsatellite mutator phenotype. *Biol Chem.* 377:675-684; Eshleman J.R., and Markowitz, S.D. (1996) Mismatch repair defects in human carcinogenesis. *Hum. Mol. Genet.* 5:1489-494). In light of this unique feature that defective MMR has on promoting MI, it is now used as a biochemical marker to survey for lack of MMR activity within host cells (Perucho, M. (1996) Cancer of the microsatellite mutator phenotype. *Biol Chem.* 377:675-684; Eshleman J.R., and Markowitz, S.D. (1996) Mismatch repair defects in human carcinogenesis. *Hum. Mol. Genet.* 5:1489-494; Liu, T., *et al.* (2000) Microsatellite instability as a predictor of a mutation in a DNA mismatch repair gene in familial colorectal cancer *Genes Chromosomes Cancer* 27:17-25).

A method used to detect MMR deficiency in eukaryotic cells is to employ a reporter gene that has a polynucleotide repeat inserted within the coding region that disrupts its reading frame due to a frame shift. In the case where MMR is defective, the reporter gene will acquire random mutations (i.e. insertions and/or deletions) within the polynucleotide

repeat yielding clones that contain a reporter with an open reading frame. We have employed the use of an MMR-sensitive reporter gene to measure for MMR activity in HBvec, HBPMS2, and HBPMS134 cells. The reporter construct used the pCAR-OF, which contains a hygromycin resistance (HYG) gene plus a β-galactosidase gene containing a 29 bp out-of-frame poly-CA tract at the 5' end of its coding region. The pCAR-OF reporter would not generate β -galactosidase activity unless a frame-restoring mutation (*i.e.*, insertion or deletion) arose following transfection. HBvec, HBPMS2, and HB134 cells were each transfected with pCAR-OF vector in duplicate reactions following the protocol described in Example 1. Cells were selected in 0.5 mg/ml G418 and 0.5mg/ml HYG to select for cells retaining both the MMR effector and the pCAR-OF reporter plasmids. All cultures transfected with the pCAR vector resulted in a similar number of HYG/G418 resistant cells. Cultures were then expanded and tested for β-galactosidase activity *in situ* as well as by biochemical analysis of cell extracts. For *in situ* analysis, 100,000 cells were harvested and fixed in 1% gluteraldehyde, washed in phosphate buffered saline solution and incubated in 1 ml of X-gal substrate solution [0.15 M NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, 0.2% X-Gal] in 24 well plates for 2 hours at 37°C. Reactions were stopped in 500 mM sodium bicarbonate solution and transferred to microscope slides for analysis. Three fields of 200 cells each were counted for blue (β-galactosidase positive cells) or white (β-galactosidase negative cells) to assess for MMR inactivation. Table 1 shows the results from these studies. While no β-galactosidase positive cells were observed in HBvec cells, 10% of the cells per field were β-galactosidase positive in HB134 cultures and 2% of the cells per field were β-galactosidase positive in HBPMS2 cultures.

Cell extracts were prepared from the above cultures to measure β-galactosidase using a quantitative biochemical assay as previously described (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641; Nicolaides, N.C., *et al.* (1992) The Jun family members, c-JUN and JUND, transactivate the human *c-myb* promoter via an Ap1 like element. *J. Biol. Chem.* 267:19665-19672). Briefly, 100,000 cells were collected, centrifuged and resuspended in 200 µls of 0.25M Tris, pH 8.0. Cells were

lysed by freeze/thawing three times and supernatants collected after microfugation at 14,000 rpms to remove cell debris. Protein content was determined by spectrophotometric analysis at OD^{280}. For biochemical assays, 20 μg of protein was added to buffer containing 45 mM 2-mercaptoethanol, 1mM $MgCl_2$, 0.1 M $NaPO_4$ and 0.6 mg/ml Chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim). Reactions were incubated for 1 hour, terminated by the addition of 0.5 M Na_2CO_3, and analyzed by spectrophotometry at 576 nm. H36 cell lysates were used to subtract out background. Figure 2 shows the β-galactosidase activity in extracts from the various cell lines. As shown, the HB134 cells produced the highest amount of β-galactosidase, while no activity was found in the HBvec cells containing the pCAR-OF. These data demonstrate the ability to generate MMR defective hybridoma cells using dominant negative MMR gene alleles.

Table 1. β-galactosidase expression of HBvec, HBPMS2 and HB134 cells transfected with pCAR-OF reporter vectors. Cells were transfected with the pCAR-OF β-galactosidase reporter plasmid. Transfected cells were selected in hygromycin and G418, expanded and stained with X-gal solution to measure for β-galactosidase activity (blue colored cells). 3 fields of 200 cells each were analyzed by microscopy. The results below represent the mean +/- standard deviation of these experiments.

Table 1.

CELL LINE	# BLUE CELLS
HBvec	0 +/- 0
HBPMS2	4 +/- 1
HB134	20 +/- 3

EXAMPLE 3: Screening strategy to identify hybridoma clones producing antibodies with higher binding affinities and/or increased immunoglobulin production.

An application of the methods presented within this document is the use of MMR deficient hybridomas or other immunoglobulin producing cells to create genetic alterations within an immunoglobulin gene that will yield antibodies with altered biochemical properties. An illustration of this application is demonstrated within this example whereby the HB134 hybridoma (see Example 1), which is a MMR-defective cell line that produces an anti-human immunoglobulin type E (hIgE) MAb, is grown for 20 generations and clones are isolated in 96-well plates and screened for hIgE binding. Figure 3 outlines the screening procedure to identify clones that produce high affinity MAbs, which is presumed to be due to an alteration within the light or heavy chain variable region of the protein. The assay employs the use of a plate Enzyme Linked Immunosorbant Assay (ELISA) to screen for clones that produce high-affinity MAbs. 96-well plates containing single cells from HBvec or HB134 pools are grown for 9 days in growth medium (RPMI 1640 plus 10% fetal bovine serum) plus 0.5 mg/ml G418 to ensure clones retain the expression vector. After 9 days, plates are screened using an hIgE plate ELISA, whereby a 96 well plate is coated with 50µls of a 1µg/ml hIgE solution for 4 hours at 4°C. Plates are washed 3 times in calcium and magnesium free phosphate buffered saline solution ($PBS^{-/-}$) and blocked in 100µls of $PBS^{-/-}$ with 5% dry milk for 1 hour at room temperature. Wells are rinsed and incubated with 100 µls of a PBS solution containing a 1:5 dilution of conditioned medium from each cell clone for 2 hours. Plates are then washed 3 times with $PBS^{-/-}$ and incubated for 1 hour at room temperature with 50 µls of a $PBS^{-/-}$ solution containing 1:3000 dilution of a sheep anti-mouse horse radish peroxidase (HRP) conjugated

secondary antibody. Plates are then washed 3 times with $PBS^{-/-}$ and incubated with 50 µls of TMB-HRP substrate (BioRad) for 15 minutes at room temperature to detect amount of antibody produced by each clone. Reactions are stopped by adding 50 µls of 500mM sodium bicarbonate and analyzed by OD at 415nm using a BioRad plate reader. Clones exhibiting an enhanced signal over background cells (H36 control cells) are then isolated and expanded into 10 ml cultures for additional characterization and confirmation of ELISA data in triplicate experiments. ELISAs are also performed on conditioned (CM) from the same clones to measure total Ig production within the conditioned medium of each well. Clones that produce an increased ELISA signal and have increased antibody levels are then further analyzed for variants that over-express and/or over-secrete antibodies as described in Example 4. Analysis of five 96-well plates each from HBvec or HB134 cells have found that a significant number of clones with a higher Optical Density (OD) value is observed in the MMR-defective HB134 cells as compared to the HBvec controls. Figure 4 shows a representative example of HB134 clones producing antibodies that bind to specific antigen (in this case IgE) with a higher affinity. Figure 4 provides raw data from the analysis of 96 wells of HBvec (left graph) or HB134 (right graph) which shows 2 clones from the HB134 plate to have a higher OD reading due to 1) genetic alteration of the antibody variable domain that leads to an increased binding to IgE antigen, or 2) genetic alteration of a cell host that leads to over-production/secretion of the antibody molecule. Anti-Ig ELISA found thatthe two clones, shown in figure 4 have Ig levels within their CM similar to the surrounding wells exhibiting ower OD values. These data suggest that a genetic alteration occurred within the antigen binding domain of the antibody which in turn allows for higher binding to antigen.

Clones that produced higher OD values as determined by ELISA were further analyzed at the genetic level to confirm that mutations within the light or heavy chain variable region have occurred that lead to a higher binding affinity hence yielding to a stronger ELISA signal. Briefly, 100,000 cells are harvested and extracted for RNA using the Triazol method as described above. RNAs are reverse transcribed using Superscript II as suggested by the manufacturer (Life Technology) and PCR amplified for the antigen binding sites contained within the variable light and heavy chains. Because of the heterogeneous nature of these

genes, the following degenerate primers are used to amplify light and heavy chain alleles from the parent H36 strain.

Light chain sense: 5'-GGA TTT TCA GGT GCA GAT TTT CAG-3' (SEQ ID NO:1)

Light chain antisense: 5'-ACT GGA TGG TGG GAA GAT GGA-3' (SEQ ID NO:2)

Heavy chain sense: 5'-A(G/T) GTN (A/C)AG CTN CAG (C/G)AG TC-3' (SEQ ID NO:3)

Heavy chain antisense: 5'-TNC CTT G(A/G)C CCC AGT A(G/A)(A/T)C-3' (SEQ ID NO:4)

PCR reactions using degenerate oligonucleotides are carried out at 94°C for 30 sec, 52°C for 1 min, and 72°C for 1 min for 35 cycles. Products are analyzed on agarose gels. Products of the expected molecular weights are purified from the gels by Gene Clean (Bio 101), cloned into T-tailed vectors, and sequenced to identify the wild type sequence of the variable light and heavy chains. Once the wild type sequence has been determined, non-degenerate primers were made for RT-PCR amplification of positive HB134 clones. Both the light and heavy chains were amplified, gel purified and sequenced using the corresponding sense and antisense primers. The sequencing of RT-PCR products gives representative sequence data of the endogenous immunoglobulin gene and not due to PCR induced mutations. Sequences from clones were then compared to the wild type sequence for sequence comparison. An example of the ability to create *in vivo* mutations within an immunoglobulin light or heavy chain is shown in figure 5, where HB134 clone92 was identified by ELISA to have an increased signal for hIgE. The light chain was amplified using specific sense and antisense primers. The light chain was RT-PCR amplified and the resulting product was purified and analyzed on an automated ABI377 sequencer. As shown in clone A, a residue –4 upstream of the CDR region 3 had a genetic change from ACT to TCT, which results in a Thr to Ser change within the framework region just preceding the CDR#3. In clone B, a residue –6 upstream of the CDR region had a genetic change from CCC to CTC, which reslts in a Pro to His change within framework region preceeding CDR#2.

The ability to generate random mutations in immunoglobulin genes or chimeric immunoglobulin genes is not limited to hybridomas. Nicolaides et al. (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype *Mol. Cell. Biol.* 18:1635-1641) has previously shown the ability to generate hypermutable hamster cells and produce mutations within an endogenous gene. A common method for producing humanized antibodies is to graft CDR sequences from a MAb (produced by immunizing a rodent host) onto a human Ig backbone, and transfection of the chimeric genes into Chinese Hamster Ovary (CHO) cells whih in turn produce a functional Ab that is secreted by the CHO cells (Shields, R.L., *et al.* (1995) Anti-IgE monoclonal antibodies that inhibit allergen-specific histamine release. *Int. Arch. Allergy Immunol.* 107:412-413). The methods described within this application are also useful for generating genetic alterations within Ig genes or chimeric Igs transfected within host cells such as rodent cell lines, plants, yeast and prokaryotes (Frigerio L, *et al.* (2000) Assembly, secretion, and vacuolar delivery of a hybrid immunoglobulin in plants. *Plant Physiol.* 123:1483-1494).

These data demonstrate the ability to generate hypermutable hybridomas, or other Ig producing host cells that can be grown and selected, to identify structurally altered immunoglobulins yielding antibodies with enhanced biochemical properties, including but not limited to increased antigen binding affinity. Moreover, hypermutable clones that contain missense mutations within the immunoglobulin gene that result in an amino acid change or changes can be then further characterized for *in vivo* stability, antigen clearance, on-off binding to antigens, etc. Clones can also be further expanded for subsequent rounds of *in vivo* mutations and can be screened using the strategy listed above.

The use of chemical mutagens to produce genetic mutations in cells or whole organisms are limited due to the toxic effects that these agents have on "normal" cells. The use of chemical mutagens such as MNU in MMR defective organisms is much more tolerable yielding to a 10 to 100 fold increase in genetic mutation over MMR deficiency alone (Bignami M, (2000) Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. *Mutat. Res.* 462:71-82). This strategy allows for the use of chemical mutagens to be used in MMR-defective Ab producing cells as a method for increasing

additional mutations within immunoglobulin genes or chimeras that may yield functional Abs with altered biochemical properties such as enhanced binding affinity to antigen, etc.

Example 4: Generation of antibody producing cells with enhanced antibody production

Analysis of clones from H36 and HB134 following the screening strategy listed above hasidentified a significant number of clones that produce enhanced amounts of antibody into the medium. While a subset of these clones gave higher Ig binding data as determined by ELISA as a consequence of mutations within the antigen binding domains contained in the variable regions, others were found to contain "enhanced" antibody production. A summary of the clones producing enhanced amounts of secreted MAb is shown in TABLE 2, where a significant number of clones from HB134 cells were found to produce enhanced Ab production within the conditioned medium as compared to H36 control cells.

TABLE 2. Generation of hybridoma cells producing high levels of antibody. HB134 clones were assayed by ELISA for elevated Ig levels. Analysis of 480 clones showed that a significant number of clones had elevated MAb product levels in their CM. Quantification showed that several of these clones produced greater than 500ngs/ml of MAb due to either enhanced expression and/or secretion as compared to clones from the H36 cell line.

Table 2. Production of MAb in CM from H36 and HB134 clones.

Cell Line	% clones > 400 ng/ml	% clones >500 ng/ml
H36	1/480 = 0.2%	0/480 = 0%
HB134	50/480 = 10%	8/480 = 1.7%

Cellular analysis of HB134 clones with higher MAb levels within the conditioned medium (CM) were analyzed to determine if the increased production was simply due to genetic alterations at the Ig locus that may lead to over-expression of the polypeptides forming the antibody, or due to enhanced secretion due to a genetic alteration affecting secretory pathway mechanisms. To address this issue, we expanded three HB134 clones that had increased levels of antibody within their CM. 10,000 cells were prepared for western blot

analysis to assay for intracellular steady state Ig protein levels (Figure 6). In addition, H36 cells were used as a standard reference (Lane 2) and a rodent fibroblast (Lane 1) was used as an Ig negative control. Briefly, cells were pelleted by centrifugation and lysed directly in 300 µl of SDS lysis buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and boiled for 5 minutes. Lysate proteins were separated by electrophoresis on 4-12% NuPAGE gels (for analysis of Ig heavy chain. Gels were electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked at room temperature for 1 hour in Tris-buffered saline (TBS) plus 0.05% Tween-20 and 5% condensed milk. Filters were probed with a 1:10,000 dilution of sheep anti-mouse horseradish peroxidase conjugated monoclonal antibody in TBS buffer and detected by chemiluminescence using Supersignal substrate (Pierce). Experiments were repeated in duplicates to ensure reproducibility. Figure 6 shows a representative analysis where a subset of clones had enhanced Ig production which accounted for increased Ab production (Lane 5) while others had a similar steady state level as the control sample, yet had higher levels of Ab within the CM. These data suggest a mechanism whereby a subset of HB134 clones contained a genetic alteration that in turn produces elevated secretion of antibody.

The use of chemical mutagens to produce genetic mutations in cells or whole organisms are limited due to the toxic effects that these agents have on "normal" cells. The use of chemical mutagens such as MNU in MMR defective organisms is much more tolerable yielding to a 10 to 100 fold increase in genetic mutation over MMR deficiency alone (Bignami M, (2000) Unmasking a killer: DNA O(6)-methylguanine and the cytotoxicity of methylating agents. *Mutat. Res.* 462:71-82). This strategy allows for the use of chemical mutagens to be used in MMR-defective Ab producing cells as a method for increasing additional mutations within immunoglobulin genes or chimeras that may yield functional Abs with altered biochemical properties such as enhanced binding affinity to antigen, etc.

Example 5: establishment of genetic stability in hybridoma cells with new output trait.

The initial steps of MMR are dependent on two protein complexes, called MutSα and MutLα (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a

Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641). Dominant negative MMR alleles are able to perturb the formation of these complexes with downstream biochemicals involved in the excision and polymerization of nucleotides comprising the "corrected" nucleotides. Examples from this application show the ability of a truncated MMR allele (PMS134) as well as a full length human PMS2 when expressed in a hybridoma cell line is capable of blocking MMR resulting in a hypermutable cell line that gains genetic alterations throughout its entire genome per cell division. Once a cell line is produced that contains genetic alterations within genes encoding for an antibody, a single chain antibody, over expression of immunoglobulin genes and/or enhanced secretion of antibody, it is desirable to restore the genomic integrity of the cell host. This can be achieved by the use of inducible vectors whereby dominant negative MMR genes are cloned into such vectors, introduced into Ab producing cells and the cells are cultured in the presence of inducer molecules and/or conditions. Inducible vectors include but are not limited to chemical regulated promoters such as the steroid inducible MMTV, tetracycline regulated promoters, temperature sensitive MMR gene alleles, and temperature sensitive promoters.

The results described above lead to several conclusions. First, expression of hPMS2 and PMS134 results in an increase in microsatellite instability in hybridoma cells. That this elevated microsatellite instability is due to MMR deficiency was proven by evaluation of extracts from stably transduced cells. The expression of PMS134 results in a polar defect in MMR, which was only observed using heteroduplexes designed to test repair from the 5' direction (no significant defect in repair from the 3' direction was observed in the same extracts) (Nicolaides *et al.* (1998) A Naturally Occurring hPMS2 Mutation Can Confer a Dominant Negative Mutator Phenotype. *Mol. Cell. Biol.* 18:1635-1641). Interestingly, cells deficient in hMLH1 also have a polar defect in MMR, but in this case preferentially affecting repair from the 3' direction (Drummond, J.T, *et al.* (1996) Cisplatin and adriamycin resistance are associated with MutLa and mismatch repair deficiency in an ovarian tumor cell line. *J. Biol. Chem.* 271:9645-19648). It is known from previous studies in both prokaryotes and eukaryotes that the separate enzymatic components mediate repair from the two different directions. Our results, in combination with those of Drummond *et al.*(Shields, R.L., *et al.* (1995) Anti-IgE monoclonal antibodies that inhibit allergen-specific histamine release. *Int.*

Arch Allergy Immunol. 107:412-413), strongly suggest a model in which 5' repair is primarily dependent on hPMS2 while 3' repair is primarily dependent on hMLH1. It is easy to envision how the dimeric complex between PMS2 and MLH1 might set up this directionality. The combined results also demonstrate that a defect in directional MMR is sufficient to produce a MMR defective phenotype and suggests that any MMR gene allele is useful to produce genetically altered hybridoma cells, or a cell line that is producing Ig gene products. Moreover, the use of such MMR alleles will be useful for generating genetically altered Ig polypeptides with altered biochemical properties as well as cell hosts that produce enhanced amounts of antibody molecules.

Another method that is taught in this application is that ANY method used to block MMR can be performed to generate hypermutablility in an antibody-producing cell that can lead to genetically altered antibodies with enhanced biochemical features such as but not limited to increased antigen binding, enhanced pharmacokinetic profiles, etc. These processes can also to be used to generate antibody producer cells that have increased Ig expression as shown in Example 4, figure 6 and/or increased antibody secretion as shown in Table 2.

In addition, we demonstrate the utility of blocking MMR in antibody producing cells to increase genetic alterations within Ig genes that may lead to altered biochemical features such as, but not limited to, increased antigen binding affinities (Figure 5A and 5B). The blockade of MMR in such cells can be through the use of dominant negative MMR gene alleles from any species including bacteria, yeast, protozoa, insects, rodents, primates, mammalian cells, and man. Blockade of MMR can also be generated through the use of antisense RNA or deoxynucleotides directed to any of the genes involved in the MMR biochemical pathway. Blockade of MMR can be through the use of polypeptides that interfere with subunits of the MMR complex including but not limited to antibodies. Finally, the blockade of MMR may be through the use chemicals such as but not limited to nonhydrolyzable ATP analogs, which have been shown to block MMR (Galio, L, *et al.* (1999) ATP hydrolysis-dependent formation of a dynamic ternary nucleoprotein complex with MutS and MutL. *Nucl. Acids Res.* 27:2325-23231).

WE CLAIM:

1. A method for making a hypermutable, antibody producing cell, comprising introducing into a cell capable of producing antibodies a polynucleotide comprising a dominant negative allele of a mismatch repair gene.
2. The method of claim 1 wherein said polynucleotide is introduced by transfection of a suspension of cells *in vitro*.
3. The method of claim 1 wherein said mismatch repair gene is *PMS2*.
4. The method of claim 1 wherein said mismatch repair gene is human *PMS2*.
5. The method of claim 1 wherein said mismatch repair gene is *MLH1*.
6. The method of claim 1 wherein said mismatch repair gene is *PMS1*.
7. The method of claim 1 wherein said mismatch repair gene is *MSH2*.
8. The method of claim 1 wherein said mismatch repair gene is *MSH2*.
9. The method of claim 4 wherein said allele comprises a truncation mutation.
10. The method of claim 4 wherein said allele comprises a truncation mutation at codon 134.
11. The method of claim 10 wherein said truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2*.
12. The method of claim 1 wherein said polynucleotide is introduced into a fertilized egg of an animal.
13. The method of claim 12 wherein the fertilized egg is subsequently implanted into a pseudo-pregnant female whereby the fertilized egg develops into a mature transgenic animal.
14. The method of claim 12 wherein said mismatch repair gene is *PMS2*.
15. The method of claim 12 wherein said mismatch repair gene is human *PMS2*.
16. The method of claim 12 wherein said mismatch repair gene is human *MLH1*.
17. The method of claim 12 wherein said mismatch repair gene is human *PMS1*.
18. The method of claim 11 wherein said mismatch repair gene is a human *mutL* homolog.
19. The method of claim 15 wherein said allele comprises a truncation mutation.
20. The method of claim 15 wherein said allele comprises a truncation mutation at codon 134.

21. The method of claim 19 wherein said truncation mutation is a thymidine at nucleotide 424 of wild-type *PMS2*.

22. The method of claim 1 wherein said capability is due to the co-introduction of an immunoglobulin gene into said cell.

23. A homogeneous culture of hypermutable, mammalian cells wherein said cells comprise a dominant negative allele of a mismatch repair gene.

24. The culture of hypermutable, mammalian cells of claim 23 wherein the mismatch repair gene is *PMS2*.

25. The culture of hypermutable, mammalian cells of claim 24 wherein the mismatch repair gene is human *PMS2*.

26. The culture of hypermutable, mammalian cells of claim 23 wherein the mismatch repair gene is *MLH1*.

27. The culture of hypermutable, mammalian cells of claim 23 wherein the mismatch repair gene is *PMS1*.

28. The culture of hypermutable, mammalian cells of claim 23 wherein the mismatch repair gene is a human *mutL* homolog.

29. The culture of hypermutable, mammalian cells of claim 23 wherein the cells express a protein consisting of the first 133 amino acids of hPMS2.

30. A method for generating a mutation in a gene affecting antibody production in an antibody-producing cell comprising:

growing a said cell comprising said gene and a dominant negative allele of a mismatch repair gene; and

testing the cell to determine whether said gene of interest harbors a mutation.

31. The method of claim 30 wherein the step of testing comprises analyzing a nucleotide sequence of said gene.

32. The method of claim 30 wherein the step of testing comprises analyzing mRNA transcribed from said gene.

33. The method of claim 30 wherein the step of testing comprises analyzing a protein encoded by the gene of interest.

34. The method of claim 30 wherein the step of testing comprises analyzing the phenotype of said gene.

35. The method of claim 30 wherein the step of testing comprises analyzing the binding activity of an antibody.

36. A method wherein a mammalian cell is made MMR defective by the process of introducing a polynucleotide comprising an antisense oligonucleotide targeted against an allele of a mismatch repair gene into a mammalian cell, whereby the cell becomes hypermutable.

37. The method of claim 36 wherein the step of testing comprises analyzing a nucleotide sequence of said gene.

38. The method of claim 36 wherein the step of testing comprises analyzing mRNA transcribed from said gene.

39. The method of claim 36 wherein the step of testing comprises analyzing a protein encoded by said gene.

40. The method of claim 36 wherein the step of testing comprises analyzing the phenotype of said gene.

41. The method of claim 36 wherein the step of testing comprises analyzing the binding activity of an antibody.

42. A method for generating a mutation in a gene affecting antibody production in an antibody-producing cell comprising:

growing said cell comprising said gene and a polynucleotide encoding a dominant negative allele of a mismatch repair gene; and

testing said cell to determine whether said cell harbors at least one mutation in said gene yielding to a new biochemical feature to the product of said gene, wherein said new biochemical feature is selected from the group consisting of over-expression of said product, enhanced secretion of said product, enhanced affinity of said product for antigen, and combinations thereof.

43. The method of claim 42 wherein the step of testing comprises analyzing the steady state expression of the immunoglobulin gene of said cell.

44. The method of claim 42 wherein the step of testing comprises analyzing steady state mRNA transcribed from the immunoglobulin gene of said cell.

45. The method of claim 42 wherein the step of testing comprises analyzing the amount of secreted protein encoded by the immunoglobulin gene of said cell.

46. The method of claim 36 wherein the cell is made by the process of introducing a polynucleotide comprising a dominant negative allele of a mismatch repair gene into a cell in the presence of DNA mutagens.

47. The method of claim 46 wherein the step of testing comprises analyzing a nucleotide sequence of an immunoglobulin gene of said cell.

48. The method of claim 46 wherein the step of testing comprises analyzing mRNA transcribed from the immunoglobulin gene of said cell.

49. The method of claim 46 wherein the step of testing comprises analyzing the immunoglobulin protein encoded by said gene.

50. The method of claim 46 wherein the step of testing comprises analyzing the biochemical activity of the protein encoded by said gene.

51. A hypermutable transgenic mammalian cell made by the method of claim 42.

52. The transgenic mammalian cell of claim 51 wherein said cell is from primate.

53. The transgenic mammalian cell of claim 51 wherein said cell is from rodent.

54. The transgenic mammalian cell of claim 51 wherein said cell is from human.

55. The transgenic mammalian cell of claim 51 wherein said cell is eukaryotic.

56. The transgenic mammalian cell of claim 51 wherein said cell is prokaryotic

57. A method of reversibly altering the hypermutability of an antibody producing cell comprising introducing an inducible vector into a cell, wherein said inducible vector comprises a dominant negative allele of a mismatch repair gene operably linked to an inducible promoter, and inducing said cell to express said dominant negative mismatch repair gene.

58. The method of claim 57 wherein said mismatch repair gene is *PMS2*.

59. The method of claim 58 wherein said mismatch repair gene is human *PMS2*.

60. The method of claim 57 wherein said mismatch repair gene is *MLH1*.

61. The method of claim 57 wherein said mismatch repair gene is *PMS1*.

62. The method of claim 57 wherein said mismatch repair gene is a human *mutL* homolog.

63. The method of claim 57 wherein said cell expresses a protein consisting of the first 133 amino acids of hPMS2.

64. The method of claim 57 further comprising analyzing the immunoglobulin protein expressed by said antibody producing cell.

65. The method of claim 64 further comprising ceasing induction of said cell, thereby restoring genetic stability of said cell.

66. A method of producing genetically altered antibodies comprising

transfecting a polynucleotide encoding an immunoglobulin protein into a cell, wherein said cell comprises a dominant negative mismatch repair gene;

growing said cell, thereby producing a hypermutated polynucleotide encoding a hypermutated immunoglobulin protein;

screening for a desirable property of said hypermutated immunoglobulin protein;

isolating said hypermutated polynucleotide; and

transfecting said hypermutated polynucleotide into a genetically stable cell, thereby producing a hypermutated antibody-producing, genetically stable cell.

67. The method of claim 66 wherein said mismatch repair gene is *PMS2.*

68. The method of claim 66 wherein said mismatch repair gene is human *PMS2.*

69. The method of claim 66 wherein said mismatch repair gene is *MLH1.*

70. The method of claim 66 wherein said mismatch repair gene is *PMS1.*

71. The method of claim 66 wherein said mismatch repair gene is a human *mutL* homolog.

72. The method of claim 66 wherein said cell expresses a protein consisting of the first 133 amino acids of hPMS2.

ABSTRACT

Dominant negative alleles of human mismatch repair genes can be used to generate hypermutable cells and organisms. By introducing these genes into cells and transgenic animals, new cell lines and animal varieties with novel and useful properties can be prepared more efficiently than by relying on the natural rate of mutation. These methods are useful for generating genetic diversity within immunoglobulin genes directed against an antigen of interest to produce altered antibodies with enhanced biochemical activity. Moreover, these methods are useful for generating antibody-producing cells with increased level of antibody production.

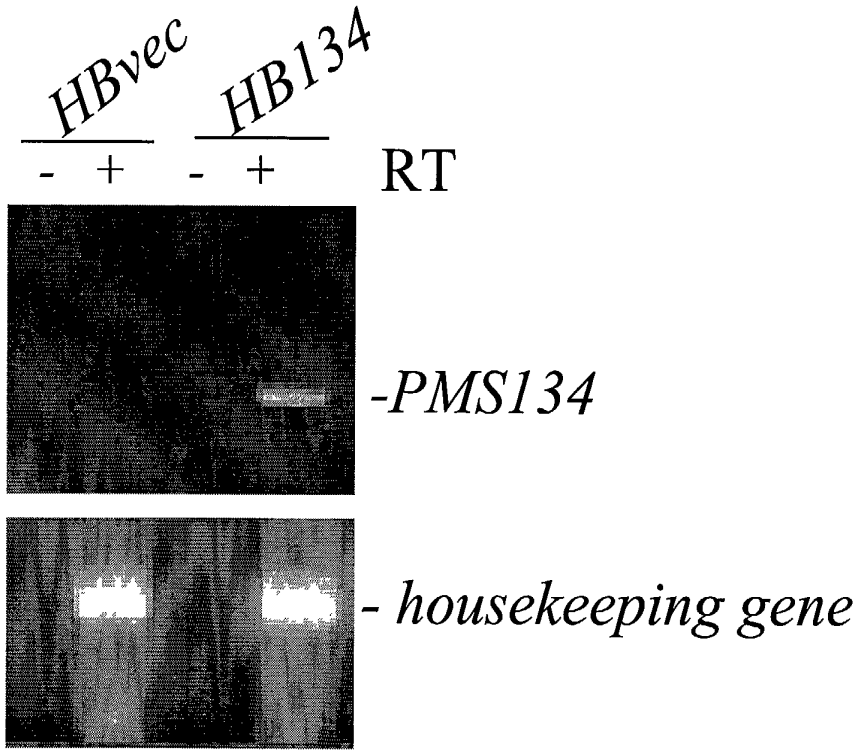


FIG. 1/6

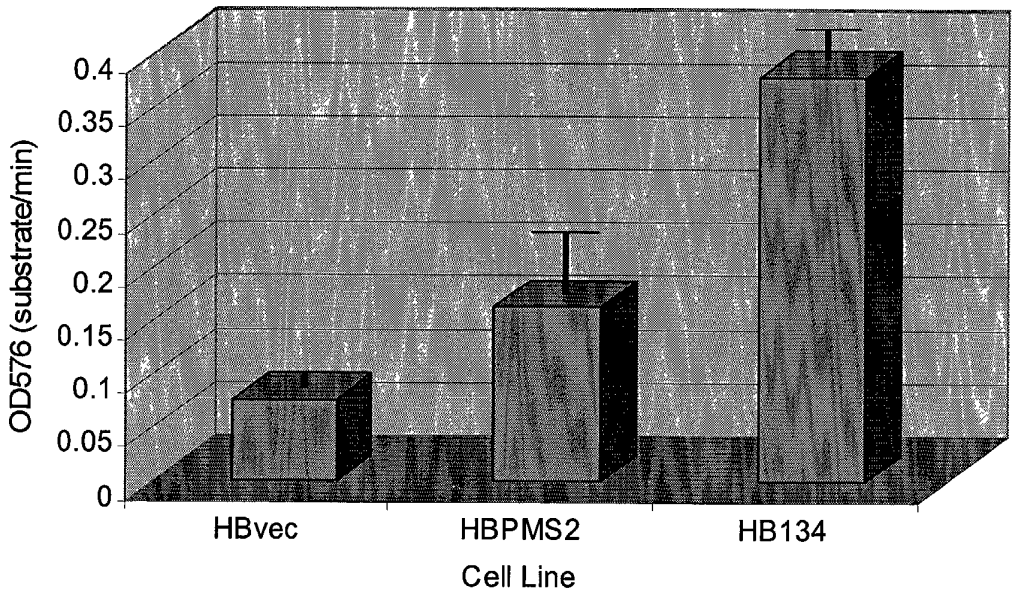


Fig. 2/6

Fig. 3/6

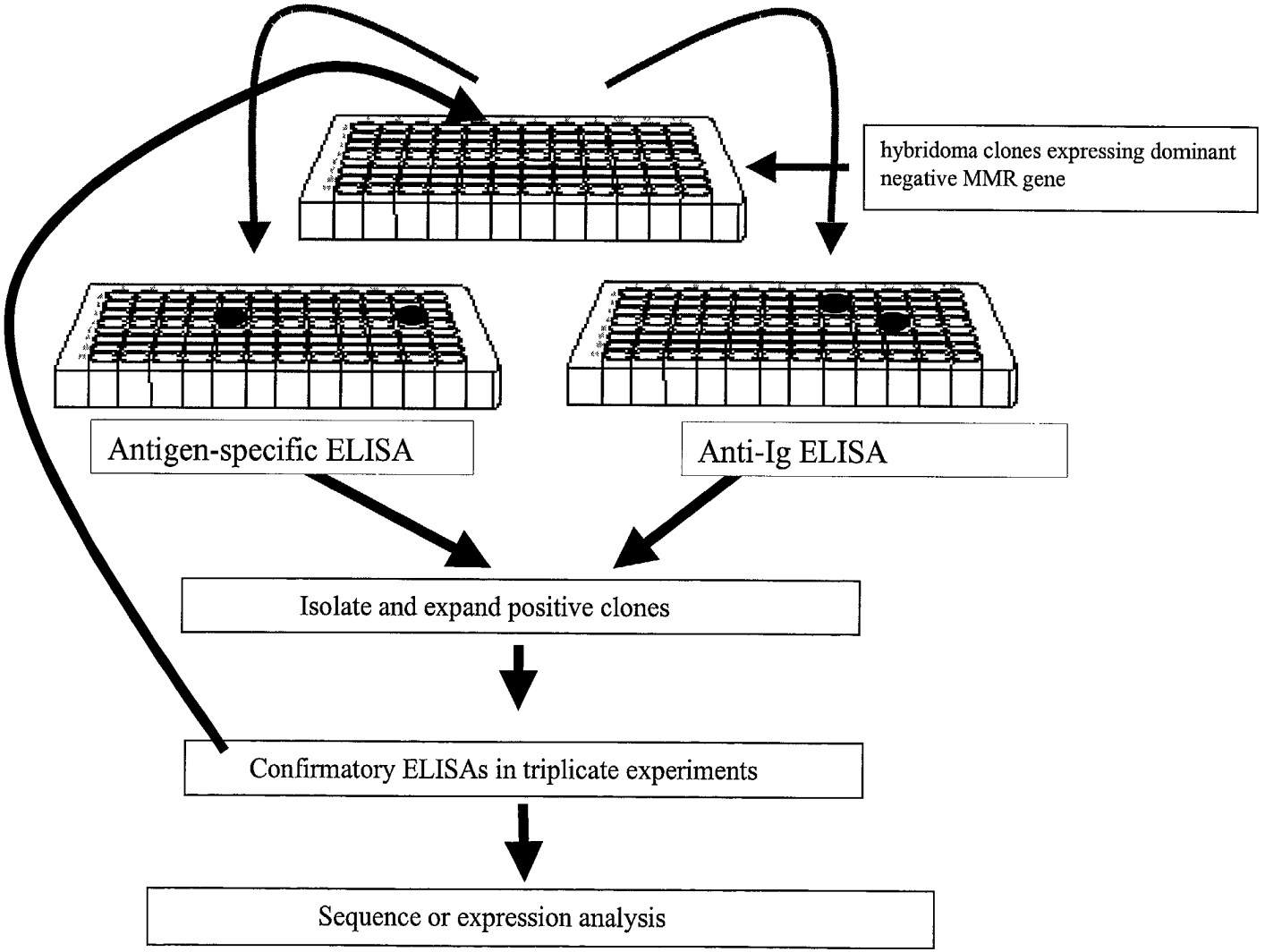
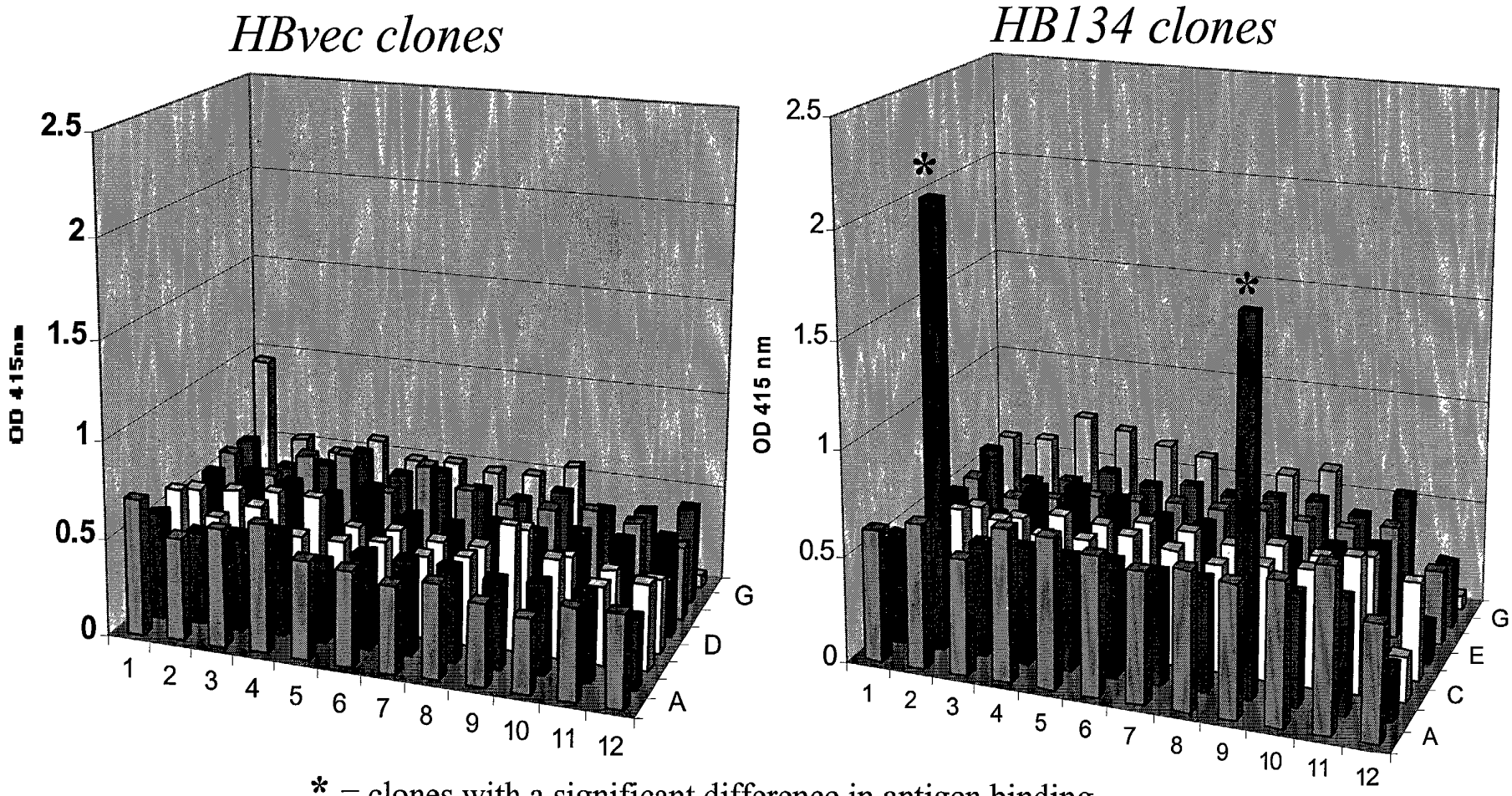
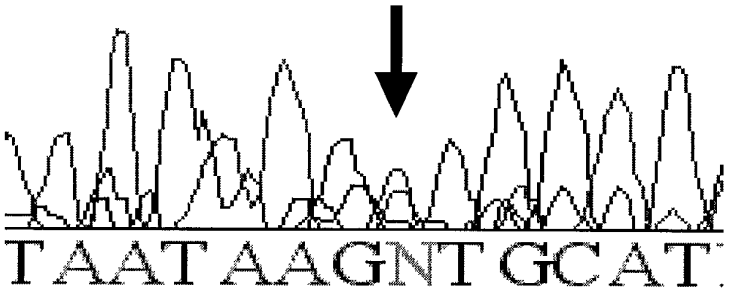


Fig.4/6



* = clones with a significant difference in antigen binding

HB134



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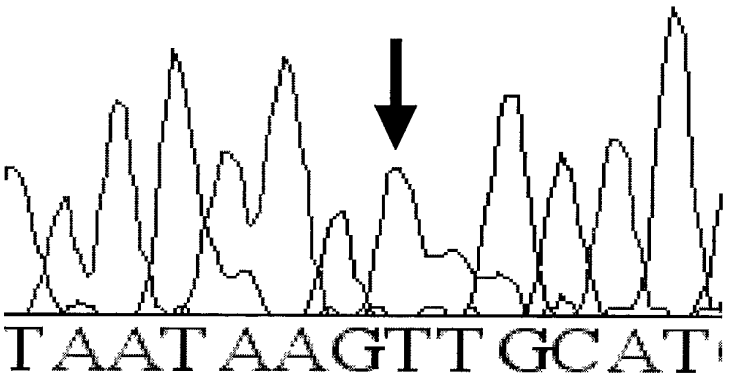

Fig. 5A/6

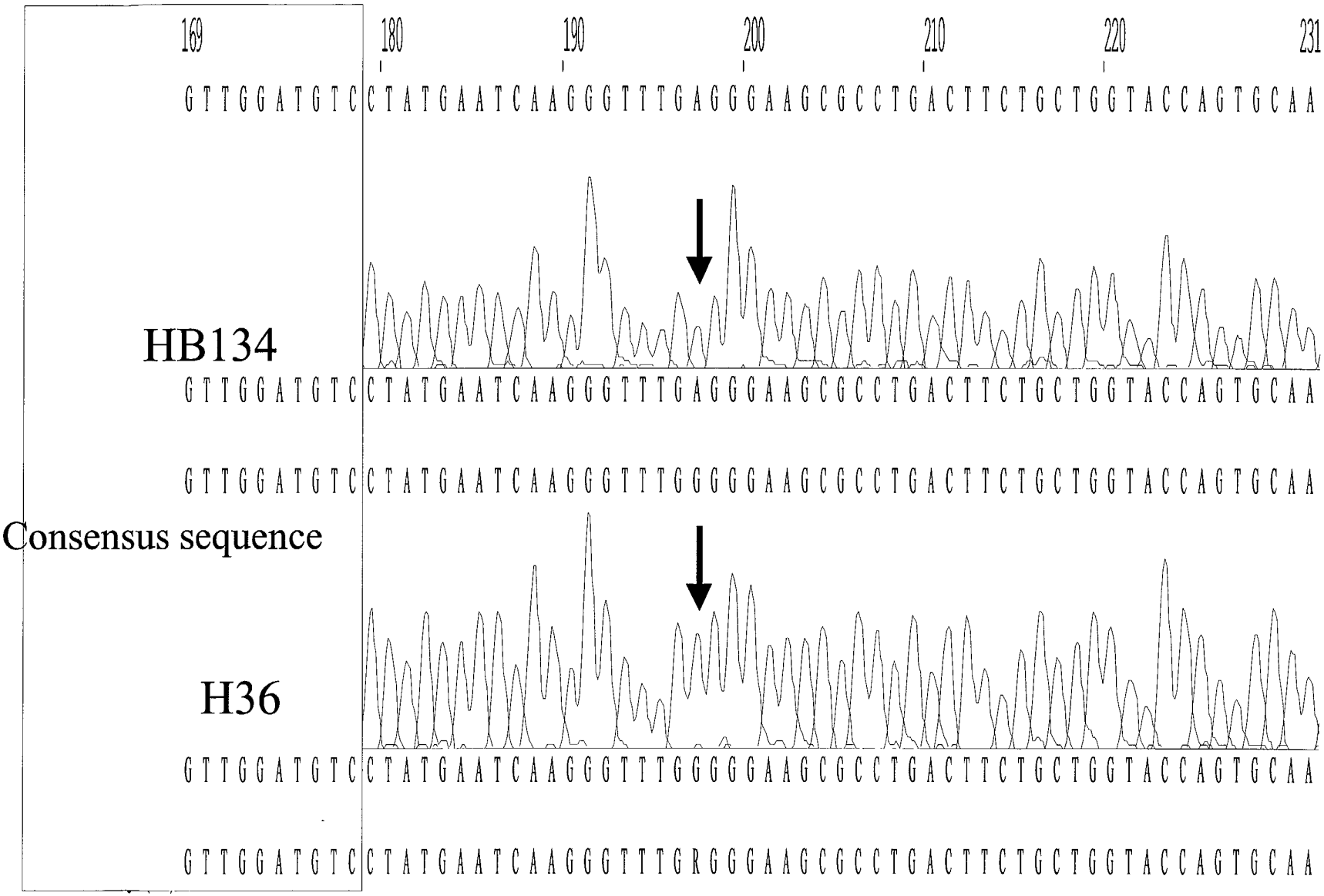

Fig. 5B/6

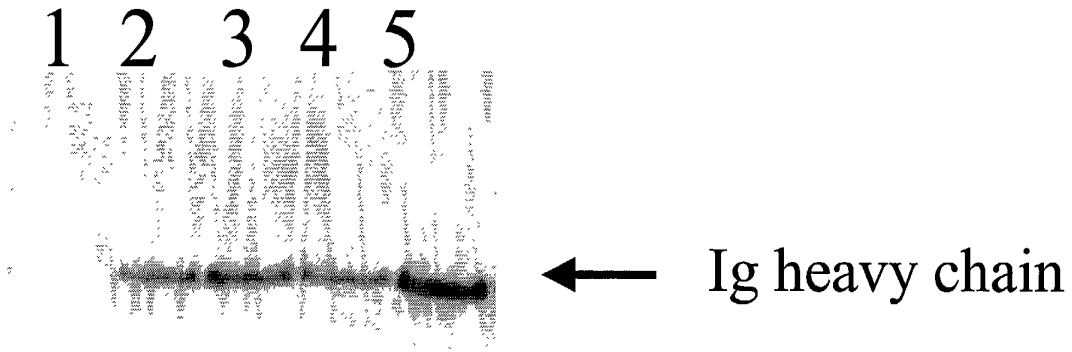

Fig. 6/6

DOCKET NO. MOR-0003 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Nicholas C. Nicolaides, Luigi Grasso, and Philip M. Sass

Group Art Unit: Not assigned

Examiner: Not assigned

For: METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODY-PRODUCING CELL LINES WITH IMPROVED ANTIBODY CHARACTERISTICS

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a

☒ Utility Patent ☐ Design Patent

is sought on the invention, whose title appears above, the specification of which:

☒ is attached hereto.

☐ was filed on _____ as Serial No. _____ .

☐ said application having been amended on _____ .

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to the patentability of this application in accordance with 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a-d) of any **foreign application(s)** for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of any application on which priority is claimed:

Priority Claimed (If X'd)	Country	Serial Number	Date Filed
☐			
☐			
☐			
☐			

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to be material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Serial Number	Date Filed	Patented/Pending/Abandoned

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below:

Serial Number	Date Filed

I hereby appoint the following persons of the firm of **WOODCOCK WASHBURN KURTZ MACKIEWICZ & NORRIS LLP**, One Liberty Place - 46th Floor, Philadelphia, Pennsylvania 19103 as attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

John W. Caldwell Reg. No. 28,937

Patrick J. Farley Reg. No. 42,524

Address all telephone calls and correspondence to:

Patrick J. Farley
WOODCOCK WASHBURN KURTZ
MACKIEWICZ & NORRIS LLP
One Liberty Place - 46th Floor
Philadelphia PA 19103
Telephone No.: **(215) 568-3100**
Facsimile No.: **(215) 568-3439**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name: Nicholas C. Nicolaides	
Mailing Address: 4 Cider Mill Court Boothwyn, PA 19061	**Signature** **Date of Signature:**
City/State of Actual Residence: Boothwyn, Pennsylvania	**Citizenship:** United States

Name: Luigi Grasso	
Mailing Address: 834 Chestnut Street, Apt#816 Philadelphia, PA 19107	_____ **Signature** **Date of Signature:** _____
City/State of Actual Residence: Philadelphia, Pennsylvania	**Citizenship:** United States

Name: Philip M. Sass	
Mailing Address: 1903 Blackhawk Circle Audubon, PA 19403	_____ **Signature** **Date of Signature:** _____
City/State of Actual Residence: Audubon, Pennsylvania	**Citizenship:** United States

Name:	
Mailing Address:	_____ **Signature** **Date of Signature:** _____
City/State of Actual Residence:	**Citizenship:** _____

DOCKET NO.: MOR-0003 **PATENT**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
Nicholas C. Nicolaides, Luigi Grasso, and
Philip M. Sass

Serial No.: Not assigned **Group Art Unit:** Not assigned

Filing Date: November 7, 2000 **Examiner:** Not assigned

For: METHODS FOR GENERATING GENETICALLY ALTERED ANTIBODY-PRODUCING CELL LINES WITH IMPROVED ANTIBODY CHARACTERISTICS

BOX SEQUENCE
Assistant Commissioner for Patents
Washington DC 20231

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 CFR §§ 1.821 THROUGH 1.825

☒ I hereby state, in accordance with the requirements of 37 C.F.R. §1.821(f), that the contents of the paper and computer readable copies of the Sequence Listing, submitted in accordance with 37 CFR §1.821(c) and (e), respectively are the same.

☐ I hereby state that the submission filed in accordance with 37 CFR §1.821(g) does not include new matter.

☐ I hereby state that the submission filed in accordance with 37 CFR §1.821(h) does not include new matter or go beyond the disclosure in the international application as filed.

☐ I hereby state that the amendments, made in accordance with 37 CFR §1.825(a), included in the substitute sheet(s) of the Sequence Listing are supported in the application, as filed, at pages _____. I hereby state that the substitute sheet(s) of the Sequence Listing does (do) not include new matter.

☐ I hereby state that the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(b), is the same as the amended Sequence Listing.

☐ I hereby state that the substitute copy of the computer readable form, submitted in accordance with 37 CFR §1.825(d), contains identical data to that originally filed.

Date: 11/7/00

Patrick J. Farley
Registration No. 42,524

Woodcock Washburn Kurtz
Mackiewicz & Norris LLP
One Liberty Place - 46th Floor
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Telephone: (215) 568-3100
Facsimile: (215) 568-3439

SEQUENCE LISTING

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Sass, Philip M

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Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser

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<213> Homo sapiens

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            20                  25                  30

Leu Asp Ala Gly Ala Thr Ser Val Asp Val Lys Leu Glu Asn Tyr Gly
        35                  40                  45

Phe Asp Lys Ile Glu Val Arg Asp Asn Gly Glu Gly Ile Lys Ala Val
    50                  55                  60

Asp Ala Pro Val Met Ala Met Lys Tyr Tyr Thr Ser Lys Ile Asn Ser
65                  70                  75                  80

His Glu Asp Leu Glu Asn Leu Thr Thr Tyr Gly Phe Arg Gly Glu Ala
                85                  90                  95

Leu Gly Ser Ile Cys Cys Ile Ala Glu Val Leu Ile Thr Thr Arg Thr
            100                 105                 110

Ala Ala Asp Asn Phe Ser Thr Gln Tyr Val Leu Asp Gly Ser Gly His
        115                 120                 125

Ile Leu Ser Gln Lys Pro Ser His Leu Gly Gln Gly Thr Thr Val Thr
    130                 135                 140

Ala Leu Arg Leu Phe Lys Asn Leu Pro Val Arg Lys Gln Phe Tyr Ser
145                 150                 155                 160

Thr Ala Lys Lys Cys Lys Asp Glu Ile Lys Lys Ile Gln Asp Leu Leu
                165                 170                 175

Met Ser Phe Gly Ile Leu Lys Pro Asp Leu Arg Ile Val Phe Val His
            180                 185                 190

Asn Lys Ala Val Ile Trp Gln Lys Ser Arg Val Ser Asp His Lys Met
        195                 200                 205

Ala Leu Met Ser Val Leu Gly Thr Ala Val Met Asn Asn Met Glu Ser
    210                 215                 220

Phe Gln Tyr His Ser Glu Glu Ser Gln Ile Tyr Leu Ser Gly Phe Leu
225                 230                 235                 240

Pro Lys Cys Asp Ala Asp His Ser Phe Thr Ser Leu Ser Thr Pro Glu
                245                 250                 255
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Arg Ser Phe Ile Phe Ile Asn Ser Arg Pro Val His Gln Lys Asp Ile
            260                 265                 270

Leu Lys Leu Ile Arg His His Tyr Asn Leu Lys Cys Leu Lys Glu Ser
        275                 280                 285

Thr Arg Leu Tyr Pro Val Phe Phe Leu Lys Ile Asp Val Pro Thr Ala
    290                 295                 300

Asp Val Asp Val Asn Leu Thr Pro Asp Lys Ser Gln Val Leu Leu Gln
305                 310                 315                 320

Asn Lys Glu Ser Val Leu Ile Ala Leu Glu Asn Leu Met Thr Thr Cys
                325                 330                 335

Tyr Gly Pro Leu Pro Ser Thr Asn Ser Tyr Glu Asn Asn Lys Thr Asp
            340                 345                 350

Val Ser Ala Ala Asp Ile Val Leu Ser Lys Thr Ala Glu Thr Asp Val
        355                 360                 365

Leu Phe Asn Lys Val Glu Ser Ser Gly Lys Asn Tyr Ser Asn Val Asp
    370                 375                 380

Thr Ser Val Ile Pro Phe Gln Asn Asp Met His Asn Asp Glu Ser Gly
385                 390                 395                 400

Lys Asn Thr Asp Asp Cys Leu Asn His Gln Ile Ser Ile Gly Asp Phe
                405                 410                 415

Gly Tyr Gly His Cys Ser Ser Glu Ile Ser Asn Ile Asp Lys Asn Thr
            420                 425                 430

Lys Asn Ala Phe Gln Asp Ile Ser Met Ser Asn Val Ser Trp Glu Asn
        435                 440                 445

Ser Gln Thr Glu Tyr Ser Lys Thr Cys Phe Ile Ser Ser Val Lys His
    450                 455                 460

Thr Gln Ser Glu Asn Gly Asn Lys Asp His Ile Asp Glu Ser Gly Glu
465                 470                 475                 480

Asn Glu Glu Glu Ala Gly Leu Glu Asn Ser Ser Glu Ile Ser Ala Asp
                485                 490                 495

Glu Trp Ser Arg Gly Asn Ile Leu Lys Asn Ser Val Gly Glu Asn Ile
            500                 505                 510
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Glu Pro Val Lys Ile Leu Val Pro Glu Lys Ser Leu Pro Cys Lys Val
        515                 520                 525

Ser Asn Asn Asn Tyr Pro Ile Pro Glu Gln Met Asn Leu Asn Glu Asp
    530                 535                 540

Ser Cys Asn Lys Lys Ser Asn Val Ile Asp Asn Lys Ser Gly Lys Val
545                 550                 555                 560

Thr Ala Tyr Asp Leu Leu Ser Asn Arg Val Ile Lys Lys Pro Met Ser
                565                 570                 575

Ala Ser Ala Leu Phe Val Gln Asp His Arg Pro Gln Phe Leu Ile Glu
            580                 585                 590

Asn Pro Lys Thr Ser Leu Glu Asp Ala Thr Leu Gln Ile Glu Glu Leu
        595                 600                 605

Trp Lys Thr Leu Ser Glu Glu Glu Lys Leu Lys Tyr Glu Glu Lys Ala
    610                 615                 620

Thr Lys Asp Leu Glu Arg Tyr Asn Ser Gln Met Lys Arg Ala Ile Glu
625                 630                 635                 640

Gln Glu Ser Gln Met Ser Leu Lys Asp Gly Arg Lys Lys Ile Lys Pro
                645                 650                 655

Thr Ser Ala Trp Asn Leu Ala Gln Lys His Lys Leu Lys Thr Ser Leu
            660                 665                 670

Ser Asn Gln Pro Lys Leu Asp Glu Leu Leu Gln Ser Gln Ile Glu Lys
        675                 680                 685

Arg Arg Ser Gln Asn Ile Lys Met Val Gln Ile Pro Phe Ser Met Lys
    690                 695                 700

Asn Leu Lys Ile Asn Phe Lys Lys Gln Asn Lys Val Asp Leu Glu Glu
705                 710                 715                 720

Lys Asp Glu Pro Cys Leu Ile His Asn Leu Arg Phe Pro Asp Ala Trp
                725                 730                 735

Leu Met Thr Ser Lys Thr Glu Val Met Leu Leu Asn Pro Tyr Arg Val
            740                 745                 750

Glu Glu Ala Leu Leu Phe Lys Arg Leu Leu Glu Asn His Lys Leu Pro
        755                 760                 765
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Ala Glu Pro Leu Glu Lys Pro Ile Met Leu Thr Glu Ser Leu Phe Asn
    770                 775                 780

Gly Ser His Tyr Leu Asp Val Leu Tyr Lys Met Thr Ala Asp Asp Gln
785                 790                 795                 800

Arg Tyr Ser Gly Ser Thr Tyr Leu Ser Asp Pro Arg Leu Thr Ala Asn
                805                 810                 815

Gly Phe Lys Ile Lys Leu Ile Pro Gly Val Ser Ile Thr Glu Asn Tyr
            820                 825                 830

Leu Glu Ile Glu Gly Met Ala Asn Cys Leu Pro Phe Tyr Gly Val Ala
        835                 840                 845

Asp Leu Lys Glu Ile Leu Asn Ala Ile Leu Asn Arg Asn Ala Lys Glu
    850                 855                 860

Val Tyr Glu Cys Arg Pro Arg Lys Val Ile Ser Tyr Leu Glu Gly Glu
865                 870                 875                 880

Ala Val Arg Leu Ser Arg Gln Leu Pro Met Tyr Leu Ser Lys Glu Asp
                885                 890                 895

Ile Gln Asp Ile Ile Tyr Arg Met Lys His Gln Phe Gly Asn Glu Ile
            900                 905                 910

Lys Glu Cys Val His Gly Arg Pro Phe Phe His His Leu Thr Tyr Leu
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Pro Glu Thr Thr
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atgctggtgc cacaagcgta gatgttaaac tggagaacta tggatttgat aaaattgagg 240
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<213> Homo sapiens

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             20                  25                  30

Thr Val Arg Leu Phe Asp Arg Gly Asp Phe Tyr Thr Ala His Gly Glu
         35                  40                  45

Asp Ala Leu Leu Ala Ala Arg Glu Val Phe Lys Thr Gln Gly Val Ile
     50                  55                  60

Lys Tyr Met Gly Pro Ala Gly Ala Lys Asn Leu Gln Ser Val Val Leu
 65                  70                  75                  80

Ser Lys Met Asn Phe Glu Ser Phe Val Lys Asp Leu Leu Leu Val Arg
                 85                  90                  95

Gln Tyr Arg Val Glu Val Tyr Lys Asn Arg Ala Gly Asn Lys Ala Ser
            100                 105                 110

Lys Glu Asn Asp Trp Tyr Leu Ala Tyr Lys Ala Ser Pro Gly Asn Leu
        115                 120                 125

Ser Gln Phe Glu Asp Ile Leu Phe Gly Asn Asn Asp Met Ser Ala Ser
    130                 135                 140

Ile Gly Val Val Gly Val Lys Met Ser Ala Val Asp Gly Gln Arg Gln
145                 150                 155                 160

Val Gly Val Gly Tyr Val Asp Ser Ile Gln Arg Lys Leu Gly Leu Cys
                165                 170                 175

Glu Phe Pro Asp Asn Asp Gln Phe Ser Asn Leu Glu Ala Leu Leu Ile
            180                 185                 190

Gln Ile Gly Pro Lys Glu Cys Val Leu Pro Gly Gly Glu Thr Ala Gly
        195                 200                 205

Asp Met Gly Lys Leu Arg Gln Ile Ile Gln Arg Gly Gly Ile Leu Ile
    210                 215                 220

Thr Glu Arg Lys Lys Ala Asp Phe Ser Thr Lys Asp Ile Tyr Gln Asp
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225                 230                 235                 240

Leu Asn Arg Leu Leu Lys Gly Lys Lys Gly Glu Gln Met Asn Ser Ala
                245                 250                 255

Val Leu Pro Glu Met Glu Asn Gln Val Ala Val Ser Ser Leu Ser Ala
            260                 265                 270

Val Ile Lys Phe Leu Glu Leu Leu Ser Asp Asp Ser Asn Phe Gly Gln
        275                 280                 285

Phe Glu Leu Thr Thr Phe Asp Phe Ser Gln Tyr Met Lys Leu Asp Ile
    290                 295                 300

Ala Ala Val Arg Ala Leu Asn Leu Phe Gln Gly Ser Val Glu Asp Thr
305                 310                 315                 320

Thr Gly Ser Gln Ser Leu Ala Ala Leu Leu Asn Lys Cys Lys Thr Pro
                325                 330                 335

Gln Gly Gln Arg Leu Val Asn Gln Trp Ile Lys Gln Pro Leu Met Asp
            340                 345                 350

Lys Asn Arg Ile Glu Glu Arg Leu Asn Leu Val Glu Ala Phe Val Glu
        355                 360                 365

Asp Ala Glu Leu Arg Gln Thr Leu Gln Glu Asp Leu Leu Arg Arg Phe
    370                 375                 380

Pro Asp Leu Asn Arg Leu Ala Lys Lys Phe Gln Arg Gln Ala Ala Asn
385                 390                 395                 400

Leu Gln Asp Cys Tyr Arg Leu Tyr Gln Gly Ile Asn Gln Leu Pro Asn
                405                 410                 415

Val Ile Gln Ala Leu Glu Lys His Glu Gly Lys His Gln Lys Leu Leu
            420                 425                 430

Leu Ala Val Phe Val Thr Pro Leu Thr Asp Leu Arg Ser Asp Phe Ser
        435                 440                 445

Lys Phe Gln Glu Met Ile Glu Thr Thr Leu Asp Met Asp Gln Val Glu
    450                 455                 460

Asn His Glu Phe Leu Val Lys Pro Ser Phe Asp Pro Asn Leu Ser Glu
465                 470                 475                 480

Leu Arg Glu Ile Met Asn Asp Leu Glu Lys Lys Met Gln Ser Thr Leu
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485 490 495

Ile Ser Ala Ala Arg Asp Leu Gly Leu Asp Pro Gly Lys Gln Ile Lys
500 505 510

Leu Asp Ser Ser Ala Gln Phe Gly Tyr Tyr Phe Arg Val Thr Cys Lys
515 520 525

Glu Glu Lys Val Leu Arg Asn Asn Lys Asn Phe Ser Thr Val Asp Ile
530 535 540

Gln Lys Asn Gly Val Lys Phe Thr Asn Ser Lys Leu Thr Ser Leu Asn
545 550 555 560

Glu Glu Tyr Thr Lys Asn Lys Thr Glu Tyr Glu Glu Ala Gln Asp Ala
565 570 575

Ile Val Lys Glu Ile Val Asn Ile Ser Ser Gly Tyr Val Glu Pro Met
580 585 590

Gln Thr Leu Asn Asp Val Leu Ala Gln Leu Asp Ala Val Val Ser Phe
595 600 605

Ala His Val Ser Asn Gly Ala Pro Val Pro Tyr Val Arg Pro Ala Ile
610 615 620

Leu Glu Lys Gly Gln Gly Arg Ile Ile Leu Lys Ala Ser Arg His Ala
625 630 635 640

Cys Val Glu Val Gln Asp Glu Ile Ala Phe Ile Pro Asn Asp Val Tyr
645 650 655

Phe Glu Lys Asp Lys Gln Met Phe His Ile Ile Thr Gly Pro Asn Met
660 665 670

Gly Gly Lys Ser Thr Tyr Ile Arg Gln Thr Gly Val Ile Val Leu Met
675 680 685

Ala Gln Ile Gly Cys Phe Val Pro Cys Glu Ser Ala Glu Val Ser Ile
690 695 700

Val Asp Cys Ile Leu Ala Arg Val Gly Ala Gly Asp Ser Gln Leu Lys
705 710 715 720

Gly Val Ser Thr Phe Met Ala Glu Met Leu Glu Thr Ala Ser Ile Leu
725 730 735

Arg Ser Ala Thr Lys Asp Ser Leu Ile Ile Ile Asp Glu Leu Gly Arg

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            740                 745                 750

Gly Thr Ser Thr Tyr Asp Gly Phe Gly Leu Ala Trp Ala Ile Ser Glu
        755                 760                 765

Tyr Ile Ala Thr Lys Ile Gly Ala Phe Cys Met Phe Ala Thr His Phe
    770                 775                 780

His Glu Leu Thr Ala Leu Ala Asn Gln Ile Pro Thr Val Asn Asn Leu
785                 790                 795                 800

His Val Thr Ala Leu Thr Thr Glu Glu Thr Leu Thr Met Leu Tyr Gln
                805                 810                 815

Val Lys Lys Gly Val Cys Asp Gln Ser Phe Gly Ile His Val Ala Glu
            820                 825                 830

Leu Ala Asn Phe Pro Lys His Val Ile Glu Cys Ala Lys Gln Lys Ala
        835                 840                 845

Leu Glu Leu Glu Glu Phe Gln Tyr Ile Gly Glu Ser Gln Gly Tyr Asp
    850                 855                 860

Ile Met Glu Pro Ala Ala Lys Lys Cys Tyr Leu Glu Arg Glu Gln Gly
865                 870                 875                 880

Glu Lys Ile Ile Gln Glu Phe Leu Ser Lys Val Lys Gln Met Pro Phe
                885                 890                 895

Thr Glu Met Ser Glu Glu Asn Ile Thr Ile Lys Leu Lys Gln Leu Lys
            900                 905                 910

Ala Glu Val Ile Ala Lys Asn Asn Ser Phe Val Asn Glu Ile Ile Ser
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Arg Ile Lys Val Thr Thr
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<213> Homo sapiens

<400> 13

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Lys Glu Met Ile Glu Asn Cys Leu Asp Ala Lys Ser Thr Ser Ile Gln
         35                  40                  45

Val Ile Val Lys Glu Gly Gly Leu Lys Leu Ile Gln Ile Gln Asp Asn
     50                  55                  60

Gly Thr Gly Ile Arg Lys Glu Asp Leu Asp Ile Val Cys Glu Arg Phe
 65                  70                  75                  80

Thr Thr Ser Lys Leu Gln Ser Phe Glu Asp Leu Ala Ser Ile Ser Thr
                 85                  90                  95

Tyr Gly Phe Arg Gly Glu Ala Leu Ala Ser Ile Ser His Val Ala His
            100                 105                 110

Val Thr Ile Thr Thr Lys Thr Ala Asp Gly Lys Cys Ala Tyr Arg Ala
        115                 120                 125

Ser Tyr Ser Asp Gly Lys Leu Lys Ala Pro Pro Lys Pro Cys Ala Gly
    130                 135                 140

Asn Gln Gly Thr Gln Ile Thr Val Glu Asp Leu Phe Tyr Asn Ile Ala
145                 150                 155                 160

Thr Arg Arg Lys Ala Leu Lys Asn Pro Ser Glu Glu Tyr Gly Lys Ile
                165                 170                 175

Leu Glu Val Val Gly Arg Tyr Ser Val His Asn Ala Gly Ile Ser Phe
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Ser Val Lys Lys Gln Gly Glu Thr Val Ala Asp Val Arg Thr Leu Pro
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Asn Ala Ser Thr Val Asp Asn Ile Arg Ser Ile Phe Gly Asn Ala Val
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Ser Arg Glu Leu Ile Glu Ile Gly Cys Glu Asp Lys Thr Leu Ala Phe
225                 230                 235                 240

Lys Met Asn Gly Tyr Ile Ser Asn Ala Asn Tyr Ser Val Lys Lys Cys
                245                 250                 255

Ile Phe Leu Leu Phe Ile Asn His Arg Leu Val Glu Ser Thr Ser Leu
            260                 265                 270

Arg Lys Ala Ile Glu Thr Val Tyr Ala Ala Tyr Leu Pro Lys Asn Thr
        275                 280                 285

His Pro Phe Leu Tyr Leu Ser Leu Glu Ile Ser Pro Gln Asn Val Asp
    290                 295                 300

Val Asn Val His Pro Thr Lys His Glu Val His Phe Leu His Glu Glu
305                 310                 315                 320

Ser Ile Leu Glu Arg Val Gln Gln His Ile Glu Ser Lys Leu Leu Gly
                325                 330                 335

Ser Asn Ser Ser Arg Met Tyr Phe Thr Gln Thr Leu Leu Pro Gly Leu
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Ala Gly Pro Ser Gly Glu Met Val Lys Ser Thr Thr Ser Leu Thr Ser
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Ser Ser Thr Ser Gly Ser Ser Asp Lys Val Tyr Ala His Gln Met Val
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Arg Thr Asp Ser Arg Glu Gln Lys Leu Asp Ala Phe Leu Gln Pro Leu
385                 390                 395                 400

Ser Lys Pro Leu Ser Ser Gln Pro Gln Ala Ile Val Thr Glu Asp Lys
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Thr Asp Ile Ser Ser Gly Arg Ala Arg Gln Gln Asp Glu Glu Met Leu
            420                 425                 430

Glu Leu Pro Ala Pro Ala Glu Val Ala Ala Lys Asn Gln Ser Leu Glu
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Gly Asp Thr Thr Lys Gly Thr Ser Glu Met Ser Glu Lys Arg Gly Pro
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Thr Ser Ser Asn Pro Arg Lys Arg His Arg Glu Asp Ser Asp Val Glu
465                 470                 475                 480

Met Val Glu Asp Asp Ser Arg Lys Glu Met Thr Ala Ala Cys Thr Pro
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Arg Arg Arg Ile Ile Asn Leu Thr Ser Val Leu Ser Leu Gln Glu Glu
            500                 505                 510

Ile Asn Glu Gln Gly His Glu Val Leu Arg Glu Met Leu His Asn His
        515                 520                 525

Ser Phe Val Gly Cys Val Asn Pro Gln Trp Ala Leu Ala Gln His Gln
    530                 535                 540

Thr Lys Leu Tyr Leu Leu Asn Thr Thr Lys Leu Ser Glu Glu Leu Phe
545                 550                 555                 560

Tyr Gln Ile Leu Ile Tyr Asp Phe Ala Asn Phe Gly Val Leu Arg Leu
                565                 570                 575

Ser Glu Pro Ala Pro Leu Phe Asp Leu Ala Met Leu Ala Leu Asp Ser
            580                 585                 590

Pro Glu Ser Gly Trp Thr Glu Glu Asp Gly Pro Lys Glu Gly Leu Ala
        595                 600                 605

Glu Tyr Ile Val Glu Phe Leu Lys Lys Lys Ala Glu Met Leu Ala Asp
    610                 615                 620

Tyr Phe Ser Leu Glu Ile Asp Glu Glu Gly Asn Leu Ile Gly Leu Pro
625                 630                 635                 640

Leu Leu Ile Asp Asn Tyr Val Pro Pro Leu Glu Gly Leu Pro Ile Phe
                645                 650                 655

Ile Leu Arg Leu Ala Thr Glu Val Asn Trp Asp Glu Glu Lys Glu Cys
            660                 665                 670

Phe Glu Ser Leu Ser Lys Glu Cys Ala Met Phe Tyr Ser Ile Arg Lys
        675                 680                 685

Gln Tyr Ile Ser Glu Glu Ser Thr Leu Ser Gly Gln Gln Ser Glu Val
    690                 695                 700

Pro Gly Ser Ile Pro Asn Ser Trp Lys Trp Thr Val Glu His Ile Val
705                 710                 715                 720
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Tyr Lys Ala Leu Arg Ser His Ile Leu Pro Pro Lys His Phe Thr Glu
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Asp Gly Asn Ile Leu Gln Leu Ala Asn Leu Pro Asp Leu Tyr Lys Val
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Phe Glu Arg Cys
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<213> Homo sapiens

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<212> PRT
<213> Homo sapiens

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Pro Ile Asp Arg Lys Ser Val His Gln Ile Cys Ser Gly Gln Val Val
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Leu Ser Leu Ser Thr Ala Val Lys Glu Leu Val Glu Asn Ser Leu Asp
35 40 45

Ala Gly Ala Thr Asn Ile Asp Leu Lys Leu Lys Asp Tyr Gly Val Asp
50 55 60

Leu Ile Glu Val Ser Asp Asn Gly Cys Gly Val Glu Glu Glu Asn Phe
65 70 75 80

Glu Gly Leu Thr Leu Lys His His Thr Ser Lys Ile Gln Glu Phe Ala
85 90 95

Asp Leu Thr Gln Val Glu Thr Phe Gly Phe Arg Gly Glu Ala Leu Ser
100 105 110

Ser Leu Cys Ala Leu Ser Asp Val Thr Ile Ser Thr Cys His Ala Ser
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Ala Lys Val Gly Thr
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<211> 426
<212> DNA
<213> Homo sapiens

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